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A VETCH ISOLATE OF CLOVER YELLOW MOSAIC VIRUS: IDENTIFICATION, INFECTIVITY ASSAY AND MULTIPLICATION

by

© DWARAPU VITAL RAO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
AND RESEARCH IN PARTIAL FULFILMENT OF THE
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A Vetch Isolate of Clover Yellow Mosaic Virus: Identification, Infectivity Assay and Multiplication submitted by Dwarapu Vital Rao in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Pathology.

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ABSTRACT

A mechanically transmissible virus isolated from field vetch (*Vicia sativa* L.) collected near the Rocky Mountain House area, Alberta, was used for all the studies described in this thesis.

The virus was identified as a distinct isolate of clover yellow mosaic virus (CYMV). In field vetch, a natural host reported for the first time in this investigation, it incited pronounced mosaic, followed by severe necrotic streaking of stems and petioles. In severity of symptoms, this isolate was intermediate between the more severe CYMV-S and the milder CYMV-H isolates of the virus.

The host range included both legumes and non-legumes, all the legumes tested to date being susceptible. Aphid transmission of the virus was negative when two species, *Acyrthosiphon pisum* (Harris) and *Myzus persicae* (Sulzer) were tested.

In vitro properties of the isolate were: DEP, 10^{-6}; TIP, 65°C; longevity, seven months. Negative staining by the leaf-dip method revealed slightly flexuous rods of uniform width and with a normal length of 511 nm. Tests for the presence of white clover mosaic virus (WCMV) yielded no evidence of this virus occurring in association with the CYMV isolate.

Different types of inclusions were demonstrated in the infected tissues of several legume hosts by light, fluorescent and electron microscopy. In light microscopy, three kinds of inclusions described

as 'amorphous', 'parallel banded' and 'hexagonal' were observed, the amorphous inclusions being most abundant. Immunofluorescence microscopy revealed the amorphous inclusions as diffuse masses of yellowish green flourescence and the parallel bands fluorescing only at the edges. In electron microscopy, aggregates of virus particles were observed in the periphery of infected cells, often orienting in certain fashions including a typical spiral form.

A procedure for purification of this isolate was developed using polyethylene glycol. The method yielded highly purified virus as checked by sucrose and cesium chloride centrifugation and by electron microscopy.

Homologous antisera to the viral immunogen of this isolate were obtained with titres of 1/2048 - 1/4096 and utilized for comparative serological tests. The isolate was serologically closely related to CYMV (including CYMV-S) and distantly related to potato virus X and WCMV as determined by the precipitin-ring tests while cross-absorption studies revealed that it and CYVM-S were serologically distinct.

The various factors affecting local lesion production were investigated with a semi-standardized assay using leaves of two-month-old Chenopodium amaranticolor Coste & Reyn. The number of lesions fluctuated least and hence most reliable at leaf positions 8 to 11 inclusive, than at other positions. Plants grown at light intensities

from 6,456 to 10,760 lux were suitable for the assay. The lesion number was significantly influenced by the post-inoculation temperature, the molarity and pH of the inoculation buffer. In general, a post-inoculation temperature of 21° - 25°C was optimal for both high lesion number and rapid lesion growth while buffer molarities from 0.005 to 0.05 and pHs from 6.5 to 8.0 were optimal for high lesion number. Dilution curves with purified virus indicated a virus concentration of 4 µg per ml to be suitable for infectivity assay.

Similarly, several factors influencing infection and multiplication of the virus were studied in cowpea mesophyll protoplast system. Protoplasts were isolated from primary leaves of the cowpea by the one-step method, inoculated with the virus and the extent of virus multiplication was determined using the fluorescent antibody technique and infectivity assay. Poly-L-ornithine in the inoculum at a concentration of 0.4 µg per ml was essential for successful infection; moreover, incubating the virus with this polycation prior to inoculation significantly increased the percentage of infected protoplasts. A virus concentration of 4 µg per ml, an inoculum pH of 6.0 and a minimal period of inoculum-protoplast contact of 15 minutes were also optimal for maximum infection. In this protoplast system, time-course studies revealed a synchronous one-step growth of virus and rapid accumulation of virus antigen was observed from 12 to 48 hours after inoculation. Under optimal conditions, infections up to 38% were achieved.

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LIST OF ABBREVIATIONS

The following abbreviations for virus names were used:

AMV, alfalfa mosaic virus

BMV, brome mosaic virus

CMV, cucumber mosaic virus

CaVX, cactus virus X

CCMV, cowpea chlorotic mottle virus

CPMV, cowpea mosaic virus

CYMV, clover yellow mosaic virus

PEMV, pea enation mosaic virus

PVX, potato virus X

TMV, tobacco mosaic virus

TNV, tobacco necrosis virus

TRV, tobacco rattle virus

TYMV, turnip yellow mosaic virus

WCMV, white clover mosaic virus

CHAPTER 1

GENERAL INTRODUCTION

Many legume viruses are economically important because of their wide distribution and virulence among various leguminous crops. In addition, the increasing significance of legume crops in human and animal nutrition and in conserving land fertility make the virus diseases extremely important in modern agriculture.

As components of forage crops, along with the grasses, legumes have contributed considerably to the total value of agricultural production in western Canada (Bratvold, 1969), and are of particular importance in Alberta (Smoliak et al., 1975; J. Willman, personal communication). As the introduction of new legume species in the province continues, the area under forage crop production has been increasing every year (Evans and Rogalsky, 1975). With increases in both the crop areas and the number of species under cultivation, there is always a danger of virus diseases among these crops. Therefore, several surveys have been conducted in recent years to investigate the magnitude of virus infections among forage legumes in the province (Hiruki and Rao, 1971; Hiruki, unpublished results).

The investigations presented in this thesis have been carried out using a new virus isolate obtained in one of the surveys. This virus was obtained from field vetch (*Vicia sativa* L.), collected near the Rocky Mountain House area, Alberta. Various studies have been made including host range, cytology, purification and serology, and the isolate has been identified as clover yellow mosaic virus (CYMV), which differs in certain serological and/or biological properties from other isolates of the virus.

CYMV is easily sap-transmissible and potentially highly infectious. It is widely distributed in western north America (Pratt, 1961; Agrawal et al., 1962a) and all legumes tested to date were found to be susceptible. It is particularly virulent on peas (Ford and Bagget, 1965) and reportedly reduced winter hardiness and yield in clovers (Pratt, 1967). Thus there is every need to control the disease. In order to formulate basic measures of control, unambiguous methods of identification are of course essential. In addition, it is very advantageous to the virologist to have available, effective multiplication and assay systems for the virus in question. An efficient method of assaying infectivity, in particular, with the use of a local lesion host, would be of immense value in the study of multiplication of the virus. Hence it is worthwhile to determine the optimal conditions related to the various factors influencing the infectivity assay.

Until 1968 virus multiplication was studied with the use of intact plant tissue which meets with an inherent disadvantage of asynchronous infection. The recent introduction of protoplast systems to achieve synchronous virus infection has greatly facilitated the study of virus multiplication (Takebe, 1975). However, it is essential to determine the various factors that would influence the virus multiplication in protoplasts before the system can be used for detailed multiplication studies. CYMV, having certain reliable requisites, serves as an excellent model for such multiplication studies. Firstly, it is stable, being easily manipulated at room temperature. Secondly, it can be easily multiplied and purified with a high yield. As a host, cowpea is ideal for multiplication studies since a large number of intact protoplasts are readily obtainable from this species. Therefore, this system is chosen as a preferred one for the study.

In summary, the objectives of this study are to obtain information pertaining to 1) the identification and characterization of the vetch isolate of CYMV, 2) the factors affecting the infectivity assay of CYMV in Chenopodium amaranticolor Coste & Reyn., and 3) the factors affecting the infection of cowpea mesophyll protoplasts with CYMV. These findings and their implications in the light of recent developments in related areas of research will be discussed.

CHAPTER 2

LITERATURE REVIEW

2.10 CLOVER YELLOW MOSAIC VIRUS

In studies of viruses occurring in clovers, Zaumeyer and Wade (1935) were probably the first to suspect the existence of two viruses in white clover. Their observations were based on differences in symptoms resulting from local and systemic infections on beans and differences in thermal inactivation points between the local and systemic components. Pierce (1935) suggested two viruses as a complex causing mosaic in red clover. One of the components inciting local necrotic lesions on broad bean was named 'broad bean local lesion virus'; the other inciting systemic infection, was named 'bean virus 2'. The first successful attempt to identify independently each of the components of a two-virus complex in white clover was made by Johnson (1942) in the U.S.A. He was able to isolate one of them which incited mottling symptoms in pea, by passage through dodder and named it 'pea mottle virus'. The other which incited streak and wilt symptoms in pea was separated by using cowpea, and was named 'pea wilt virus'. Matsulevich (1956) reported a red clover mosaic in the Ukraine and indicated the presence of Johnson's pea wilt and pea mottle viruses as a complex in the infected red clover samples.

The earlier reports, largely based on host range studies and *in vitro* properties, lack the more reliable types of information that have since become available with improved techniques in plant virology. Thus Bos et al. (1959), when presenting the first detailed characterization of white clover mosaic virus (WCMV), showed that the virus had features in common with Johnson's pea wilt--pea mottle complex, and demonstrated the inadequacy of Johnson's distinction. However, in Canada, Pratt (1961) was able to distinguish serologically two viruses isolated from naturally infected white clover. The first virus of the complex was proved to be the 'white clover mosaic virus' (WCMV). It was separated by early recovery from the infected trifoliate leaves of cowpea. This virus incited solid necrotic lesions on broad bean followed by systemic mosaic and did not incite any local lesions on *C. amaranticolor* or *Gomphrena globosa*. The second virus was separated by passage through *C. amaranticolor* and *Antirrhinum majus*. This was named 'clover yellow mosaic virus' (CYMV). Pratt (1961) suggested the names WCMV and CYMV as synonyms for Johnson's (1942) 'pea wilt' and 'pea mottle' viruses respectively. Further distinction of the viruses was made possible by the determination of the differences in particle size (Pratt and Reichmann, 1961), physical property (Pratt, 1961), electrophoretic mobility (Pratt and Reichmann, 1961) and molecular weight of the protein subunits (Koenig et al., 1970).

With regard to geographical distribution, CYMV has been reported only from North America (Pratt, 1961; Agrawal et al., 1962a;

Ford and Bagget, 1965; Bos, 1973; Welsh et al., 1973). On the other hand, WCMV has been prevalent in Europe and North America (Bos et al., 1959, 1960b; Bancroft et al., 1960), and the reports of Fry (1959), Fry et al. (1960), and Latch and Procter (1966) indicate that it is also distributed widely in the pastures of New Zealand.

The present review deals with the literature concerned with various aspects of investigations on CYMV.

The host range of CYMV includes both legumes and non-legumes. All legumes tested have been susceptible to the virus, and susceptible non-legumes include the local lesion hosts *C. amaranticolor*, *Chenopodium quinoa*, *G. globosa* and *Phaseolus aureus*; the systemic hosts *A. majus*, *Cucumis sativus*, *Spinacia oleracea*, *Stellaria media*, and *Tetragonia expansa*. Welsh et al. (1973) isolated CYMV from apple trees.

Although CYMV is easily transmitted by sap, limited studies of Pratt (1961) with the pea aphid (*Acyrthosiphon pisum*) and the clover aphid (*Anuraphis bakeri*) gave negative results. Transmission through dodder was successful with some isolates only, and a low percentage of seed transmission was reported by Hampton (1963).

For most of the CYMV isolates the thermal inactivation point (TIP) was determined to be between 58 - 60°C, and the dilution end point (DEP) lay in the neighbourhood of 10^{-4}, while longevity *in vitro* was greater than six months with a significant decrease of infectivity between 6 and 12 months (Pratt 1961). The principal exception to these findings was the apple isolate of CYMV (Welsh et al.,1973), which had a TIP of 52°C, a DEP of 10^{-8} and a longevity in vitro of nine weeks.

Several methods have been developed for the purification of CYMV, using infected pea tissue in most cases as the starting material. The conventional procedure of differential centrifugation without the use of organic solvents for preliminary clarification was used by Pratt (1961) and Agrawal et al. (1962b), while Bercks and Brandes (1963) reported preliminary clarification of the sap with ether-carbon tetrachloride, followed by differential centrifugation. An alternative method of sap clarification was described by Welsh et al. (1973) utilizing chloroform in high molarity buffer, or the liquid-nitrogen procedure. They reported that these two procedures were the most suitable among the methods tested for the apple isolate of CYMV. Purcifull and Shepherd (1964) initially precipitated virus at its isoelectric point, resuspended by readjusting to a higher pH, and subjected the resulting suspension to high-speed centrifugation after dialysis. Another purification procedure has been developed by Ford (1973) using infected pea roots. He claimed that purification from roots eliminated one cycle of differential centrifugation by avoiding the necessity of chlorophyll removal.

Pratt and Reichmann (1961) reported that CYMV sedimented in the analytical centrifuge as a single peak with a sedimentation value of 121 S, when the virus was prepared in 0.1 M phosphate buffer, pH 7.0. A similar peak with a value of 122 S was reported by Welsh et al. (1973) for the apple isolate when clarified sap, prepared in 0.01 M neutral phosphate buffer, was used. Ford (1973), on the other hand, using Pratt's isolate (Pratt, 1961) consistently obtained three peaks, with S values of 188, 203 and 282 when the virus was prepared in 0.01 M neutral phosphate buffer. However, in 0.05 M sodium borate buffer, pH 8.4, two peaks with S values of 135 and 182, and an occasional third peak at 250 S was again obtained.

CYMV was found to produce abundant inclusion bodies in its host tissue (Pratt, 1966). Christie (1967), using different stain combinations to demonstrate these in the CYMV-infected pea tissue, reported two types of inclusions. One type exhibited a dark parallel banding and the other appeared as an aggregate of dense bodies surrounding an amorphous area. Electron microscopic studies have shown that the densly stained bands of the banded inclusions and the dense bodies of the other inclusion type were themselves composed of numerous virus particles; the lightly stained areas consisted of thread like particles intermingled with host ribosomes and membranous structures. Some aggregates exhibited a spiral structure (Purcifull et al., 1966). Recently, Hiruki et al. (1976) have demonstrated the presence of CYMV aggregates in the transfer cells of the infected pea tissue. Schlegel and Delisle (1971) moreover, reported, by immuno-autoradiography, the presence of a large number of antigenic inclusions prior to the formation of virus particles in the cytoplasm and vacuoles of broad bean leaf tissue. These inclusions, seen in the earlier stages of infection, were labelled heavily over their entire structure whereas the mature viral inclusions formed later were labelled only at the edges.

Titres of antisera up to 1/2560 were produced against CYMV antigen (Pratt, 1961; Bercks and Brandes, 1963) and Bercks (1963) reported a titre as high as 1/32,768 when the antibody was precipitated using 40% ammonium sulfate. In tube-precipitin tests, Pratt (1961) demonstrated a flocculent gelatinous type of precipitate when the reaction was positive, and in agar-gel diffusion tests, precipitin lines were formed close to antiserum wells after a relatively long incubation time (Ford, 1964). However, CYMV was strongly immunogenic when it was degraded with 0.1 M ethanolamine, pH 10.5. Strong precipitin lines were readily formed in agar-gel diffusion tests when degraded fragments of CYMV were used as antigen (Purcifull and Shepherd, 1964).

2.20 INFECTIVITY ASSAY

A convenient method of assaying the relative concentration of a virus inoculum is to test its infectivity on a local lesion host. Before 1929, a single infected plant served as a unit of infection and the concentration of a virus was determined by counting the number of systemically infected plants (McKinney, 1927). In 1929, however, Holmes reported that species of Nicotiana, in particular N. glutinosa, produced discrete local lesions after inoculation of leaves with TMV.

The number of local lesions produced was roughly proportional to the concentration of the virus tested, so that the single lesion has been considered as the unit of infection. This facilitated the assay of virus infectivity on a quantitative basis, since there are many advantages of using a local lesion host in virus studies.

A variety of local lesion hosts have been described in the literature for several viruses. These mostly belong to the families Aizoaceae, Amaranthaceae, Chenopodiaceae, Polygonaceae, Portulacaceae and Caryophyllaceae (Hollings, 1959, 1966). For quantitative testing, several methods of local lesion assay have been developed. Since the discovery by Samuel and Bald (1933) that there was less variation in the number of lesions produced between the opposite halves of the same leaf than between different leaves, the half-leaf method has been widely used in assays when two or more samples are to be compared. The number of leaves used in an assay depends on the host species and the number of samples to be compared, while systematic procedures have been followed for applying inocula to the leaves to minimize statistical variability. Various experimental designs and their implications for virus assay have been adequately reviewed by Roberts (1964) and Kleczkowski (1967).

Among the factors that affect the local lesion assay, virus inoculum should be considered first. Although Ragetli et al. (1973) reported significant increases in the number of local lesions using dry inocula obtained from freeze-dried powders of infected tissue, many workers have used liquid inocula. In general, infected crude sap with suitable dilution has frequently been used as virus inoculum, the concentration of which is one of the major factors governing the accuracy of the assay. For TMV (Best, 1937) and several other viruses, dilution curves have been worked out. Such curves may consist of three parts: 1) a relatively flat region representing higher concentrations, where a change in virus concentration has little effect on lesion number, 2) a slope region where a change in virus concentration accompanies a proportional change in lesion number and, 3) another flat region at higher dilutions. Upon comparing two samples of inocula, the two points representing numbers of local lesions/dilution should lie on the slopes of the curves, otherwise the comparisons of infectivity may lead to several-fold errors.

Dilution curves, besides having practical value as described above are also important in theoretical considerations of the quantitative basis of virus infection. Lauffer and Price (1945) and Bald (1950) theoretically interpreted the virus dilution curves. They pointed out that these curves show the best fit to a Poisson distribution, assuming that a single particle initiates a lesion. Using a similar assumption, modified to take into account the potential infectible nature of each cell, Furomoto and Mickey (1967a, b) constructed theoretical single-hit curves for TMV from the data obtained from 30 experiments. Nevertheless, serious deviations from single-hit kinetics, if they occur, may be interpreted as indicating specific kinds of interference. For example, Fulton (1962) using sour cherry necrotic ringspot and prune dwarf viruses, and Yarwood (1964) using peach ring-spot virus, obtained abnormally steep dilution curves, and Fulton (1962) suggested the reason was possibly a requirement of more than one virus particle to initiate infection. Such curves are in fact expected for viruses carrying a divided genome, where more than one virus particle is necessary to initiate infection or to achieve increased infectivity (Wood and Bancroft, 1965; van Kammen, 1968; van Vloten-Doting et al., 1968). Thus van Vloten-Doting et al. (1968) have reported abnormally steep curves for AMV, a virus with a divided genome.

Plant viruses require wounding of the plant tissue for successful entry and infection of plant cells. The use of an abrasive to injure the cells to establish virus infection was recognized as early as 1930 by Fajardo who used sand in the transmission of a bean virus. Carborundum, the trade name for silicon carbide, was first used by Rawlins and Tompkins (1936) in their virus inoculations, and since then it has been widely used in mechanical transmission of plant viruses. Another commonly used abrasive is Celite (Kalmus and Kassanis, 1945). The size (Kalmus and Kassanis, 1945), and number (Beraha et al., 1955) of abrasive particles and sequence of their use in inoculation (Yarwood, 1968) influences the efficacy of the abrasive.

Inoculum is uniformly applied on the surface of the leaf with the finger (Allington and Laird, 1954) or with various tools depending on the choice of the investigator and the amount of inoculum used. Brushes (Takahashi, 1947), spatulas (Samuel, 1931), cloth pads (Ross, 1953), spray guns (Timian et al., 1956), and cotton swabs (Hiruki, 1970) have been used. An interesting variation has been tried by Lamborn et al. (1971) using ultrasonication to inoculate bean leaves with TMV. They reported an 88-fold increase in the number of lesions over the conventional finger rubbing method.

To stabilize the infectivity of viruses, various additives either alone or in combination with other chemicals have been used. Thornberry (1935) first showed the positive effect of phosphate buffer as an additive on TMV infectivity and Yarwood (1952, 1962) reported it to be significant with bean plants inoculated with several viruses in his detailed study on the 'phosphate effect'. The role of phosphate in increasing lesion number is not conclusively known; it may act on the virus (Diachun and Valleau, 1950), or on the host (Yarwood 1952, 1962). Another additive, sucrose, at 2 M concentration, has also been found effective in the transmission of a number of viruses (Grant and Corbett, 1960; Davis and Whitcomb, 1967). However, Yarwood (1971) reported that the effect of sucrose under routine labatory conditions was negligible and that its effect depended on the host plant species used and/or the presence or absence of other additives in the inoculum.

A number of other additive compounds increased the local lesion number incited by several viruses (Hecht-Poinar and Yarwood, 1966; Yarwood and Fulton, 1967; Joshi and Thornberry, 1968; Yarwood 1969). More recently, 2-thiouracil has been shown to cause significant increases in the size and number of local CCMV lesions on soybean leaves treated with the additive after inoculation (Kuhn, 1971).

Several environmental factors affecting the physiological state of the host plants also affect the infectivity assay. The most important of these are water, light and darkness, temperature,and nutrition. Well-watered plants are generally more suitable for bioassay. Significant increases in local lesion number have been reported with several viruses on well-watered plants over the sparsely watered controls (Tinsley, 1953; Singh, 1969). In addition, bean plants grown at a high relative humidity before inoculation showed increased susceptibility to infection by TNV (Kimmins, 1967; Kimmins and Leitz, 1967). On the other hand, sustained moisture during the post-inoculation period seems to reduce the degree of infection. Thus while Holmes (1929) found that a brief washing of leaves immediately after inoculation increased the lesion number, Yarwood (1955) has demonstrated that either a sustained post-inoculation wash of more than 10 seconds or incubation in a moist chamber decreased the infectivity. Quick drying, preferably within 1 second after inoculation, in fact was found to increase lesion number (Yarwood, 1963, 1973). Yarwood (1955) suggested the deleterious effect of water in the case of sustained washing, was due to dilution of ions necessary for attachment and penetration of virus.

With regard to the influence of pre-inoculation darkness vs. light on the susceptibility of plants, there are several reports in the literature demonstrating increases in host plant susceptibility due to dark treatment before inoculation. (Bawden and Roberts, 1947, 1948; Ross, 1953; Trujillo and Saettler, 1972; Hiruki, 1975). The increased susceptibility, however, was not consistent in certain instances, and the effect seems to be influenced by varying periods of light before (Helms and McIntyre, 1967a, b) or after inoculation (Matthews 1953b) or by the time of year (Wiltshire, 1956). The findings of Matthews (1953a) and Yarwood (1957) in particular, that plants show greater susceptibility when inoculated in the afternoon after an extended period of light rather than in the morning after a long dark period suggest that the effect of darkness vs. light on host susceptibility may be complex. Post-inoculation dark treatment decreased the number of lesions (Huguelet, 1964; Huguelet and Hooker, 1966; Wilcoxson and El-kandelgy, 1966) an effect that could be counteracted by detaching the leaves and keeping them in a nutrient solution supplemented with glucose or sucrose (Huguelet, 1964; Huguelet and Hooker, 1966).

Post-inoculation light treatment within certain intensity limits, appears to have an overall positive effect in increasing the number of lesions incited by several viruses (Yarwood, 1957; Huguelet and Hooker, 1966; Rosenkranz and Hagedorn, 1964). The positive light-induced susceptibility seems also to depend on the wavelength of the light used. For example, Coast and Chant (1970) reported that growing plants under green light increased the susceptibility of N. glutinosa to infection by TMV when compared between red or blue lights. Furthermore, higher light intensities changed the appearance of lesions incited by PVX in G. globosa and also caused the production of spontaneous virus-like lesions (Francki, 1967).

A variety of studies have been made on the effects of temperature on the host susceptibility before and after inoculation with viruses. Two different responses of the host plants due to pre-inoculation temperatures have been demonstrated; abnormally high temperature (Best, 1936; Kassanis, 1952) or abnormally low temperature of about 10°C (Harrison, 1956) significantly increased the local lesion number. Higher post-inoculation temperatures within certain limits either increased (Ross, 1953) or decreased (Rosenkranz and Hagedorn, 1964) the number of local lesions. The lesion size (Kassanis, 1952; Rosenkranz and Hagedorn, 1964) and the rate of appearance of lesions (Hiruki, 1975) increased under similar conditions. However, the length of time required for the appearance of lesions decreased (Hiruki, 1975) or did not decrease (Wolffgang, 1970) depending on the systems used.

Hot water treatment, as demonstrated by Yarwood (1952) by dipping bean leaves in hot water at 45°C for 0.5-1 minute, greatly increased the number of lesions incited by TMV. Foster and Ross

(1975a, b) demonstrated TMV-induced local lesions by hot water treatment of Turkish tobacco, which under normal conditions of infection does not develop lesions. Wu et al. (1969) correlated the size of lesions and the rate of callose deposition on the walls of cells adjacent to the necrotic cells. They interpreted increases in the number of lesions observed after heat treatment as being due to the increases in size and visibility of minute lesions resulting from decreased rates and amounts of callose deposition.

An interesting approach to heat treatment was made by Yarwood et al. (1962) who reported the production of translocated stimuli due to heat treatment. They showed that heating a primary bean leaf increased the susceptibility of the opposite leaf as much as 300-fold, and Nienhaus and Yarwood (1963) after further studies explained such increases to be related primarily to activation of infection leading to necrosis rather than initiation of infection.

The influence of host nutrition on susceptibility to virus infection has received a limited amount of attention. The nutritional state of the plant is known, however, not only to affect the distinctness of lesions formed but also the number of lesions incited. Thus distinct, purple local lesions were incited by TYMV on Chinese cabbage plants grown under nitrogen deficient conditions as compared with the chlorotic lesions produced under normal nutritional conditions (Diener and Jenifer, 1964). Bawden and Kassanis (1950), using a range of fertilizer treatments, found furthermore that TMV lesions incited in Nicotana tabacum and N. glutinosa leaves were increased in number after the plants were subjected to both nitrogen and phosphorus treatments, and concluded that these treatments affected host susceptibility through the host growth. These conclusions appear to be supported by Spencer (1935a) who observed increased susceptibility with nitrogen application, and Foster (1967), who reported increases in CMV lesion number on C. amaranticolor grown under balanced levels of nutrients. However, potassium at concentrations lower than those that favor the growth of the plants increased the local lesion number (Spencer 1935b; Allington and Laird, 1954) indicating the influence of the nutrient on host susceptibility is at least not through the host growth. Growing plants in manganese solutions increased the susceptibility to virus infections (Dhaliwal and Rudd, 1970; Singh et al., 1974).

2.30 PROTOPLASTS IN VIRUS INFECTION AND MULTIPLICATION STUDIES

One of the problem areas in the study of plant virus replication is the non-synchronous infection commonly observed when an intact leaf, representing a considerable depth of cells, is inoculated with a virus. Initially, the number of cells infected is very small and the virus replication occurs at different times in cells of the same leaf because of the variable secondary spread. This asynchrony makes it difficult to study precisely one complete replicative cycle of the virus, hence there is a need for a system where the number of plant cells initially infected is substantially high and there is no secondary spread. This is possible only when a large number of individual cells or protoplasts can be isolated and inoculated simultaneously *in vitro*. The use of cells, however, meets with the disadvantage of having cell walls which are a barrier for the entry of virus particles. Therefore, to obtain virus infected cells, it is necessary to isolate them from plants which have been infected systemically. (Zaitlin, 1959; Takebe et al., 1968). Protoplasts have a unique advantage over the single cells in that they can readily be inoculated after isolation. However, the following assumptions have to be made when protoplasts are used: 1) the protoplasts isolated are physiologically active and potentially susceptible to virus infection and 2) no secondary spread occurs through the culture medium during their incubation.

The first conclusive evidence that TMV can infect and multiply in a substantially large number of protoplasts of tobacco was presented by Takebe's group (Takebe and Otsuki, 1969; Aoki and Takebe, 1969), while Cocking (1966) was the first to use enzymatically isolated protoplasts in virus infection studies. Their work considerably stimulated interest in this area, and since then there have been many reports on successful infection of protoplasts with plant viruses.

At present, at least 10 plant viruses have been shown to infect and multiply in protoplasts. These include rod, spherical and bacilliform viruses (Takebe, 1975). Plant viruses carrying divided genomes have also been used (Motoyoshi et al., 1973b, 1974a, 1975; Motoyoshi and Hull, 1974; Hibi et al., 1975; Kubo et al., 1975b). Infection studies with viral RNA have been made with few viruses (Aoki and Takebe, 1969; Motoyoshi et al., 1973b), and the percentage of infection has been relatively lower than with the whole virus.

The majority of workers have used tobacco mesophyll protoplasts in their infection studies. The protoplasts may be isolated enzymatically, by either 1) the two-step method, or 2) the one-step method. The former method originated from Takebe et al. (1968). In the first step, the leaf material is digested with a commerical pectinase, which attacks the pectin of the middle lamella and allows individual cells of the spongy and palisade tissues to emerge into the surrounding hypertonic medium. Spongy cells are discarded and the palisade cells are retained in the medium as they are believed to be more suitable for virus infection. In the second step, a cellulase is used to digest the cell walls to give rise to spherical, membrane-bound protoplasts. In the one-step method the pectinase and cellulase are combined in the medium so that the digestion of the middle lamella and cell walls takes place simultaneously (Kassanis and White, 1974; Hibi et al., 1975). The two-step method is relatively gentle and results in a homogeneous population of protoplasts, whereas the one-step method, though relatively simple, gives rise to a heterogeneous population of protoplasts.

The inoculation of protoplasts with viruses has often been carried out in the presence of citrate buffer. Kubo et al. (1974, 1975b, 1976), however, reported phosphate buffer to be better than citrate buffer for the infection of tobacco protoplasts by TRV. Either the direct or indirect method may be used for inoculation of protoplasts. In the indirect method (Takebe and Otsuki, 1969), the protoplasts are suspended in mannitol and immediately mixed with an appropriate volume of the inoculum to give the final required concentrations. In the direct method (Motoyoshi et al., 1974b), however, the freshly sedimented protoplasts are suspended directly in the inoculum. Motoyoshi et al. (1974b) have claimed higher percentages of infection by the direct method than by the indirect method in the CCMV-tobacco protoplast system, whereas Kubo et al. (1975b), reported little difference between the two methods using TRV-tobacco protoplasts.

For culturing protoplasts after inoculation, a majority of workers used the medium of Takebe et al. (1968) with minor modifications. This medium is simple and can support a rapid multiplication of viruses in protoplasts. Moreover, it does not allow the protoplasts to divide, which is extremely important in studies of one-step growth.

Various antibiotics and antifungal compounds have been used in culture media to prevent bacterial and fungal contamination during incubation. The results of Motoyoshi et al. (1974b) and Kassanis et al. (1975) point out, however, the necessity of using these compounds with caution because the nature and concentration of some of these compounds seem to affect virus multiplication.

There are mainly four factors that influence the frequency of infection and multiplication of the virus. These are: 1) the condition of the source plant, 2) the pH of the inoculation buffer, 3) the presence or absence of poly-L-ornithine in the inoculum and 4) the concentration of virus.

The growth condition of the source plants is an important factor in the successful isolation and infection of protoplasts. For example, the age of tobacco plants used for the isolation of mesophyll protoplasts greatly influences the susceptibility of protoplasts to infection with CCMV (Mctoyoshi et al., 1974b). The choice of leaf material fit for isolation of stable protoplasts has been described for greenhouse-grown plants by Watts et al. (1973). Kubo et al. (1975a) reported that tobacco plants grown under defined conditions were even more reliable sources of protoplasts for virus infection.

The pH of the buffer is important in infection because there appears to be a critical pH optimum at which maximum virus infection and replication occurs. The pH optimum for the majority of viruses tested is around 5.0, while that for PVX (Otsuki et al., 1974), TRV (Kubo et al., 1975b) and TYMV (Renaudin et al., 1975) is higher.

Poly-L-ornithine, a linear polymer of a basic amino acid, was first used by Takebe and Otsuki (1969) in their TMV-protoplast system, and was one of the reasons for their success in obtaining high frequencies of infection. Other polymers, such as poly-L-arginine and poly-D-lysine, have been used (Motoyoshi et al., 1974b) but they were not as effective as poly-L-ornithine. At the present time, this polymer seems to be the choice of a majority of workers. The concentrations used were in the range between 0.5 µg per ml and 2 µg per ml, except in one case where an unusually high concentration has been used (Shalla and Petersen, 1973). Poly-L-ornithine is required by a majority of negatively charged viruses (Takebe and Otsuki 1969; Otsuki and Takebe, 1973; Motoyoshi et al., 1973b, 1975; Otsuki et al., 1974; Renaudin et al., 1975) but not by positively charged viruses(Motoyoshi and Hull, 1974; Motoyoshi et al., 1974a). Although it stimulates infection in the latter, the actual role of poly-L-ornithine in infection seems to be a complex one; the hypothesis that initial virus uptake is aided by the stimulation of pinocytosis has been proposed by Cocking (1970) and supported by the findings of Hibi and Yora (1972), Otsuki et al. (1972) and Honda et al. (1974). Contrasting evidence has, however, been presented by Burgess et al. (1973a, b), who propose that virus uptake could take place by plasmalemma lesions apparently caused by poly-L-ornithine. Whatever the mechanism may be, the presence of poly-L-ornithine is obligatory for the majority of viruses so far studied.

With regard to the concentration of the inoculum, a linear relationship exists between the concentration of virus and the percentage of infection up to a certain point, beyond which infection tends to level off (Hibi et al., 1975) or decrease (Takebe and Otsuki, 1969; Otsuki and Takebe, 1973; Otsuki et al., 1974). An important factor influencing the percentage of infection is the specific infectivity of the virus itself.

There are three principal methods that are commonly used to assess the virus concentration in the protoplasts: 1) fluorescent antibody technique 2) infectivity assay and 3) electron microscopy. All three techniques have advantages and disadvantages of their own, but when used together they complement each other in furnishing substantial information about virus replication. In addition, several other methods are used, including the serological assay (Coutts et al., 1972; Shepard, 1975), sucrose density gradient centrifugation (Motoyoshi et al., 1974a; Motoyoshi and Hull, 1974; Renaudin et al., 1975) and radioisotope technique (Motoyoshi et al., 1973a, Motoyoshi and Hull, 1974)

The fluorescent antibody technique involves the use of γ-globulin which is immunologically specific for a given virus and is chemically combined with fluoroscein isothiocyanate. The labelled antibody acts as a useful marker by combining with the antigen accumulated in the protoplasts (Coons, 1958; Otsuki and Takebe, 1969). The technique is simple and very convenient once the labelled antibody is prepared. It is widely used and appears to be the best choice among other techniques, especially when a virus multiplies poorly in the protoplast (Motoyoshi and Hull, 1974).

Fluorescence is usually observed in the early stages of multiplication as tiny spots dispersed in the cytoplasm of the infected protoplasts. These spots may grow into giant masses or crystalloid structures by the end of the exponential period of multiplication (Otsuki and Takebe, 1969; Hibi et al., 1975). In addition, fluorescence has also been observed in the nuclei of tobacco protoplasts infected with CMV (Otsuki and Takebe, 1973) and PEMV (Motoyoshi and Hull, 1974), indicating the accumulation of viral antigen in the nucleus.

An infectivity assay using a local lesion host is the most efficient way to estimate relative virus activity, provided the local lesion host is highly sensitive. A typical infectivity pattern for synchronous infection may be generalized as follows. The virus bound to the protoplast usually shows some infectivity immediately after inoculation. This is followed by a 'lag', which lasts up to 8 hours, indicated by very low or no infectivity. Later, a linear rise in infectivity occurs, usually extending up to 24 hours, followed by a slower increase up to 72 hours after inoculation. Variations from the typical growth, however, have been shown by several workers. For example, Motoyoshi et al. (1973b) using CCMV, Otsuki and Takebe (1973) using CMV reported no infectivity at zero hour after inoculation, the reason being thought to be a low sensitivity of the assay host. A slow rise in infectivity has been observed by Kubo et al. (1975b) with the TRV-tobacco protoplast system, and by Shalla and Petersen (1973) with the PVX-tobacco protoplast system. However, in the case of TRV, low incubation temperatures or a multigenomic nature of the virus has been claimed by the former authors as the cause for the slow rise.

Electron microscopy was used either to demonstrate the presence of progeny virus at the end of a replication cycle (Hibi et al., 1975; Kubo et al., 1975b) or to follow the time course of replication. The results obtained by electron microscopy of TMV, CMV and PVX from the time course study fit well with those obtained by the fluorescent antibody technique and infectivity assays (Otsuki et al., 1972; Honda et al., 1974, 1975).

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF A VETCH ISOLATE OF CLOVER YELLOW MOSAIC VIRUS

3.10 INTRODUCTION

Field infections of vetch with legume viruses other than CYMV have been reported in the literature (Ford, 1965; McCord and Gudauskas, 1968; Tolin and Miller, 1975). The present investigation describes the natural infection of CYMV on field vetch (V. sativa) and detailed characterization of this isolate of the virus.

3.20 MATERIALS AND METHODS

3.21 Virus and virus isolates

The virus (hereafter called 'vetch isolate') used in this study, was originally isolated from field vetch (V. sativa) collected near the Rocky Mountain House area, Alberta. Unless otherwise stated, the virus maintained in pea (Pisum sativum L. 'Alaska') was used for subsequent experiments.

The CYMV isolates and other viruses which were used in comparative studies of symptomatology and serology were obtained from the following sources: CYMV - S isolate from Dr. R. Stace-Smith of Agriculture Canada, Vancouver; PVX from Dr. C. Hiruki, Edmonton; WCMV from Dr. R.J. Shepherd, Davis, California, and CYMV-H isolate (originally obtained from Dr. R.O. Hampton, Corvallis, Oregon) from Dr. J.C. Tu, Edmonton.

3.22 Growth conditions

Unless otherwise stated, all plants were grown in a greenhouse at 25°C. Light intensity ranged between 15,000 and 20,000 lux in summer, and in fall and winter the light period was adjusted to 16 hours per day by providing artificial illumination with fluorescent and incandescent lights at about 16,000 lux.

3.23 Host range

The crude sap obtained from the original diseased sample multiplied in field vetch was used as inoculum in the host range studies, carried out on plants maintained in a greenhouse at standard conditions. The plants tested included 29 species representing 19 genera and nine families. Inoculations were done by stroking leaves uniformly dusted with 22 µm (600 - mesh) Carborundum with a cotton swab (Q-tips, Chesebrough-Pond's (Canada) Ltd., Toronto) that had been dipped in the sap inoculum. At least 10 plants were used for each plant species and the experiments were repeated several times using uninoculated plants as controls. Both the inoculated and uninoculated leaves of the plants were subjected to virus recovery tests on _C. amaranticolor_.

3.24 Insect transmission

The pea aphid, *Acyrthosiphon pisum* (Harris) and the green peach aphid, *Myzus persicae* (Sulzer) were used in insect transmission. The aphids were pre-starved for 30 minutes, allowed an acquisition access period of 5 - 30 minutes, and transferred to healthy pea plants at the rate of 2 - 5 per plant. After an inoculation test period of 8 hours, the inoculated plants were sprayed with the insecticide Pirimor (Plant Products Co., Port Credit, Ontario), and kept in a greenhouse. A total of 20 plants were used for inoculation by each species of aphid. Fifteen days after inoculation feeding, a sample leaf from each plant was removed, ground in phosphate buffer and the sap extract was used to inoculate *C. amaranticolor*.

3.25 *In vitro* properties

The properties of the virus *in vitro*, i.e., DEP, TIP and longevity, were determined by standard methods (Bos et al., 1960a) and infectivity assays were made on the leaves of *C. amaranticolor*. All tests were repeated at least two times. Dilutions of the sap were made in 0.025 M phosphate buffer, pH 7.0. For TIP and longevity tests, a sap dilution of 10^{-2} was used.

3.26 Electron microscopy

Leaf-dip preparations were made from infected pea leaves, freshly cut surfaces of which were dipped into a drop of glass-distilled water on Formvar-coated grids. Excess water was removed and the grid samples were negatively stained with 2% aqueous phosphotungstic acid (PTA), pH 7.0, the extra stain from the grid being removed with a piece of filter paper. After air drying, these were examined with a Philips Model 200 transmission electron microscope (60 and 80 kV). Purified preparations and their fractions obtained after sucrose density gradient centrifugation were similarly stained with PTA. Polysterene latex spheres (Ernest F. Fullman Inc., Schenectady, New York) with an average diameter of 312 nm were added to the preparations at one-to-five dilution as an internal size standard.

3.27 Cytology - virus inclusions

For light microscopic observations of inclusions, four legume hosts were used. These were: field vetch (V. sativa), pea (P. sativum L. 'Alaska'), broad bean (Vicia faba L. 'Broad Windsor') and cowpea (Vigna sinensis Savi 'Black Eye'). The leaves were inoculated with crude sap of infected peas approximately two weeks after germination. Sampling was done one and two weeks after inoculation. Epidermal strips obtained from the underside of the systemically infected leaves were used except for cowpea, where the epidermal strips were taken from the inoculated primary leaves.

The stains employed to differentiate virus inclusions were:

1) Phloxine B 2) Azure B 3) Iodine-potassium iodide. Stains 1 and 2 were prepared at 1% concentration in the solvent described by Christie (1967). Iodine-potassium iodide was prepared by dissolving 2 gm of potassium iodide and 0.2 gm iodine in 100 ml of distilled water.

The epidermal strips were immersed in the stain solutions for 10-15 minutes, rinsed and mounted in distilled water for examination by light microscopy. The epidermal strips from the leaves of uninoculated plants served as controls.

For immunofluorescence studies of inclusion bodies, epidermal strips obtained from inoculated leaves of cowpea were cut into 2 mm squares and placed on glass slides smeared with Haupt's adhesive. These slides were then immersed in 95% ethanol for 15 minutes and washed in phosphate-buffered saline (PBS). Fluorescent antibody staining and observation of the fluorescence were subsequently carried out according to the procedure given in Chapter 5 (page 91).

For transmission electron microscopic observations, systemically infected leaves of field vetch were sampled two weeks after inoculation. The sample tissues, cut in 2 mm squares, were fixed in 3% Formalin - glutaraldehyde mixture (1:1, V/V) in 0.1 M phosphate buffer, pH 7.0 at 4°C for 7 hours. The fixed materials were then washed and post-fixed in 2% osmium tetroxide for 5 hours, dehydrated through a graded series of ethanol, taken to propylene oxide and embedded in Araldite. Sections were cut on a Reichert ultramicrotome using a diamond knife and stained with 2% aqueous uranyl acetate for 2 hours followed by 2 - 5 minutes with lead citrate.

3.28 Purification

Infected Alaska pea tissue showing marked mosaic symptoms was used for purification. Frozen tissue was homogenized in a Waring blendor with cold 0.1 M phosphate buffer, pH 7.0 (2 ml per gm) containing 0.5 - 1% ascorbic acid, and the homogenate was strained through a double layer of cheesecloth and subjected to low-speed centrifugation. The virus was precipitated from the clarified supernatant by adding 8 gm of polyethylene glycol (PEG, mol. wt. 6000) and 0.6 gm of sodium chloride per 100 ml of extract. After a 2-hour incubation at 4°C, the precipitated virus was collected by low-speed centrifugation, and the pellets were suspended in 0.025 M phosphate buffer, pH 7.0 containing 0.001 M disodium ethylenediamine-tetraacetate (EDTA) and held overnight at 4°C. The suspension was centrifuged at low-speed, and the supernatant, containing the virus, was then subjected to two cycles of differential centrifugation and the resulting virus pellets were dissolved in the above mentioned buffer (Figure 1).

Low-speed centrifugations were carried out in a Sorvall RC2-B refrigerated centrifuge for 15-20 minutes at 11,000-12,000 g.

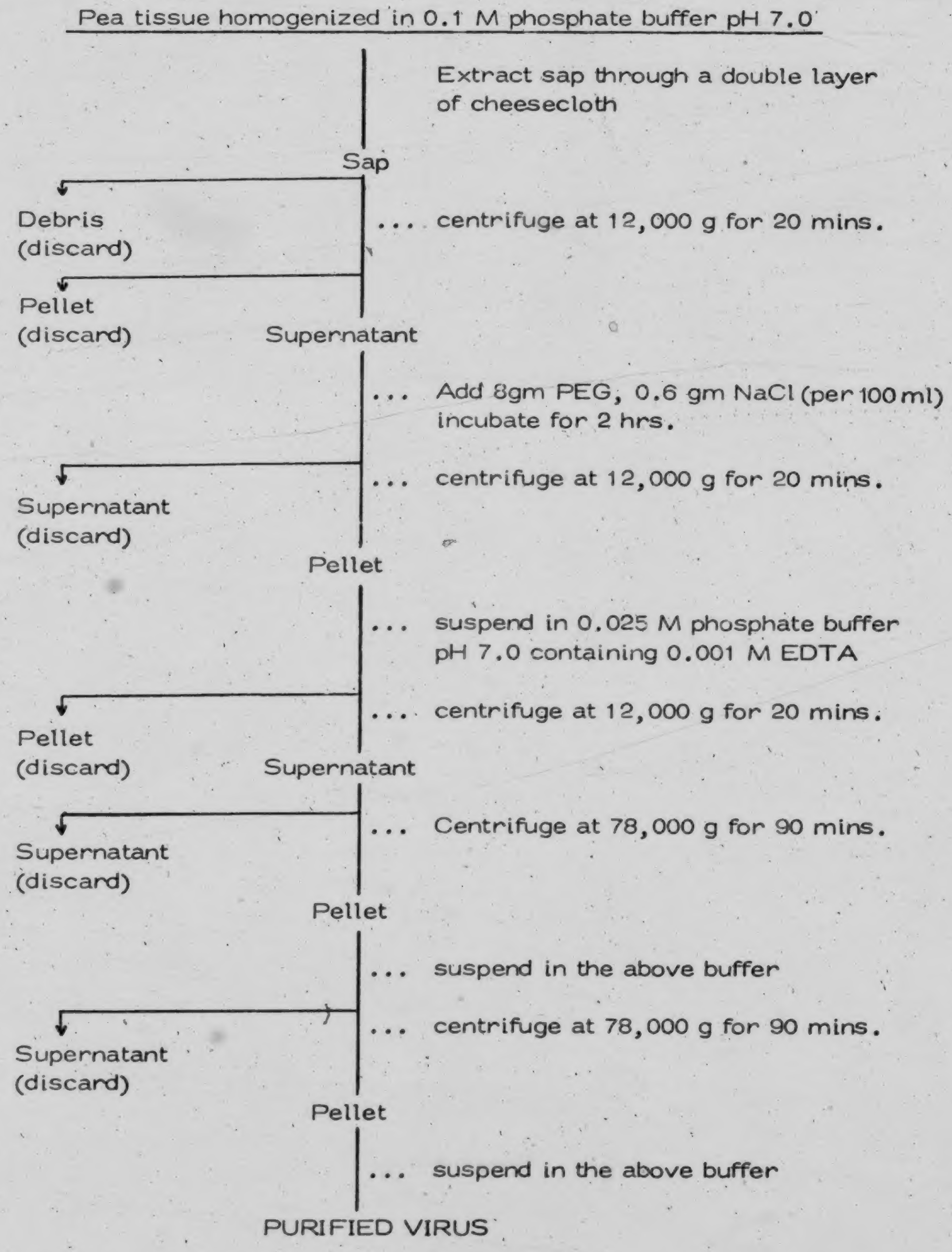
High-speed centrifugations were performed for 90 minutes at 78,000 g in a Spinco Model L-4 preparative ultracentrifuge.

A. Sucrose density gradient centrifugation

Linear sucrose gradients of 10-40% were prepared in 0.025 M phosphate buffer, pH 7.0, and the purified virus preparation was layered on top of each gradient. The gradients were then centrifuged at 64,000 g for 90 minutes in a Spinco 25.1 swinging bucket rotor and fractionated with an ISCO Model D density gradient fractionator. One ml fractions were collected in separate tubes, dialyzed against 0.005 M phosphate buffer, pH 7.0, and measured for absorbance at 260 nm with a Hitachi Perkin-Elmer 139 UV spectrophotometer. The infectivity of each fraction was assayed on C. amaranticolor after suitable dilution. Those infective fractions that showed higher UV absorption at 260 nm were used for electron microscopy and for determining the UV absorption spectrum of the virus in the 220-315 nm wave length range.

B. Cesium chloride centrifugation

To determine the purity of the isolated virus, cesium chloride density gradients of 20-40% were prepared in 0.01 M tris buffer containing 0.1 M sodium chloride and 1 mM EDTA, and purified virus at 3-5 mg per ml was layered on the top of the gradient. Centrifugation was performed at 100,000 g in rotor 50.1 of a Beckman



Pea tissue homogenized in 0.1 M phosphate buffer pH 7.0

Extract sap through a double layer of cheesecloth

Sap

... centrifuge at 12,000 g for 20 mins.

Debris (discard)

Pellet (discard)

Supernatant

... Add 8gm PEG, 0.6 gm NaCl (per 100 ml) incubate for 2 hrs.

... centrifuge at 12,000 g for 20 mins.

Supernatant (discard)

Pellet

... suspend in 0.025 M phosphate buffer pH 7.0 containing 0.001 M EDTA

... centrifuge at 12,000 g for 20 mins.

Pellet (discard)

Supernatant

... Centrifuge at 78,000 g for 90 mins.

Supernatant (discard)

Pellet

... suspend in the above buffer

... centrifuge at 78,000 g for 90 mins.

Supernatant (discard)

Pellet

... suspend in the above buffer

PURIFIED VIRUS

Figure 1. Flow diagram for the purification of the vetch isolate of clover yellow mosaic virus.

Model L-2 preparative ultracentrifuge for 20 hours.

3.29 Analytical ultracentrifugation

To determine the sedimentation coefficient, the purified virus samples were used with or without sucrose density-gradient centrifugation. In the former case, an infective fraction (fraction 7) obtained from three sucrose gradient columns was pooled and subjected to high-speed centrifugation at 78,000 g for 90 minutes. The virus, obtained as a small pellet, was suspended in 0.01 M phosphate buffer, pH 7.0, and a sample was used to determine the S value by analytical ultracentrifugation in a Beckman Model E ultracentrifuge equipped with high-intensity optical system, monochromator assembly and photoelectric scanner. The progression of the sedimenting boundary under 30,000 g at 20°C was recorded at 4-minute scanning intervals. The sedimentation coefficients were calculated for standard conditions.

3.30 Dry weight determination and correlation of absorbance

The purified sample was dried in a freeze drier (Virtis Company Inc., Gardiner, New York) and the dry weight of the sample was determined with a monopan balance (Sartorius-werke, Göttingen, West Germany). The dried sample was dissolved in an aliquot of 0.01 M phosphate buffer, pH 7.0, and the suspension was serially diluted. The absorbance at 260 nm was then determined with a spectrophotometer for each dilution and an absorbance value was calculated for the virus concentration at 1 mg per ml.

3.31 Serology

A. Preparation of antigens

CYMV-S and PVX were multiplied in Alaska pea and tomato plants respectively. In some serological tests, purified virus obtained by the PEG method described previously was used. Dilutions were made in 0.025 M phosphate buffer, pH 7.0, and an optimal dilution of 0.1-0.3 mg per ml was used. In others, the crude sap, prepared by grinding 1gm of tissue in 9 ml of 0.025 M phosphate buffer, pH 7.0, was used. This was strained through a double layer of cheesecloth, centrifuged at low-speed, and the supernatant diluted as required.

B. Preparation of antisera

To prepare homologous antiserum, San Juan rabbits were given a total of nine intravenous injections at one-week intervals with the purified virus at the rate of 3-5 mg per injection. Bleeding was performed at the end of each week to determine the titre of the antiserum, and final bleeding was carried out one week after the last injection. For serological tests, the homologous antiserum and other antisera described in Table 1 were diluted in 0.85% sodium chloride (saline).

C. Serological technique

The antibody titres were determined by precipitin-ring tests carried out in 3 mm-diameter test tubes. Each dilution of antiserum was added to a height of 15 mm, and the same volume of a constant dilution of antigen was gently layered on the top. The tubes were incubated at 37°C in a water bath and observed after 30 minutes, 1 hour and 3 hours. The appropriate separate controls with saline, normal serum and healthy sap were included.

D. Serological relationships

The antisera prepared against certain member viruses of the potex group (Fenner, 1976) were used to determine the possible relationship of the vetch isolate. Table 1 shows the sources of antisera obtained.

For cross-absorption, the purified virus was mixed with the antiserum at a two-to-one volume ratio, and kept at 37°C for 2 hours. This mixture was kept overnight at 4°C and centrifuged at low-speed, after which the supernatant was removed and tested with the purified virus at optimal dilution.

3.40 RESULTS

3.41 Host range and symptoms

A. Symptoms in field vetch

When two-week-old field vetch plants were sap-inoculated, vein-clearing appeared in 10 days on the younger leaves and was soon

Table 1. The names and sources of antisera used in the identification of the vetch isolate of clover yellow mosaic virus

Antiserum	Code	Name of the supplier
CYMV[a]	CYMV-S	R. Stace-Smith, Research Station, Agriculture Canada, Vancouver.
CYMV[a]	PVAS-56	American Type Culture Collection, Rockville, Maryland.
CYMV[a]	PVAS-67	American Type Culture Collection, Rockville, Maryland.
WCMV[b]	PVAS-36	American Type Culture Collection, Rockville, Maryland.
PVX[c]	PV-54	American Type Culture Collection Rockville, Maryland.
CaVX[d]	PVAS-79	American Type Culture Collection, Rockville, Maryland.

[a] CYMV, clover yellow mosaic virus.

[b] WCMV, white clover mosaic virus.

[c] PVX, potato virus X.

[d] CaVX, cactus virus X.

followed by mosaic and necrotic spotting (Plate 1A and B). Seven to eight weeks after inoculation, the infected plants displayed drastic reduction in leaf size with irregular leaf laminae (Plate 1A) and pronounced necrotic streaking of the stems and petioles. Wilting and death of the necrotic plants was observed consistently during winter months.

To compare the symptoms incited by CYMV isolates, crude sap (1/10 dilution) of pea plants infected with each isolate was used. The sap-inoculation was made on two-week-old field vetch plants. The three isolates were distinguished on the basis of the degree of severity of symptoms on field vetch. The vetch isolate incited systemic necrosis without early defoliation. Symptoms incited by the CYMV-S isolate were conspicuous necrosis of leaves, petioles and stems, which resulted in rapid drying and defoliation. The CYMV-H isolate incited symptoms of general mosaic, although the necrosis was less severe in the advanced stages of infection (Plate 1B).

B. Other hosts

Table 2 summarizes the results of host range studies on the vetch isolate. The non-legumes tested and found not to be susceptible were: *Brassica oleracea* L., *Datura stramonium* L., *Lilium formosanum* Sapf., *Lycopersicon esculentum* Mill., *Nicotiana clevelandii* A. Gray, *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. 'Bright Yellow', *Raphanus sativus* L., and *Zinnia elegans* Jacq.

Table 2. Reactions of plants to inoculations with the vetch isolate of clover yellow mosaic virus

Host plant	Symptoms[a]		Virus recovered from[b]	
			Inoculated leaves	Uninoculated leaves
Legumes				
Glycine max Merr.	Mo		+	+
Medicago sativa L.	Mo		+	+
Phaseolus aureus Roxb.	LL, Mo		+	+
P. vulgaris L				
'Red Kidney'	Mo	(1D[c])	+	+
'Pinto'	Mo		+	+
Pisum sativum L.				
'Alaska'	Mo	(1C)	+	+
'Little Marvel'	Mo		+	+
'Dwarf Telephone'	Mo		+	+
'Wisconsin Perfection'	Mo, N, Str.		+	+
'Perfected Wales'	Mo, N, Str.		+	+
Trifolium hybridum L.	Mo	(2C)	+	+
T. pratense L.	Mo		+	+
T. repens L.	Mo	(2D, E)	+	+
Vicia faba L.				
'Broad Windsor'	LL, Mo, N, Str.	(2A, B)	+	+
'Ackerpearl'	LL, Mo, N		+	+
V. sativa L.	Mo, N, Str.	(1A, B)	+	+
V. villosa Roth	Mo, N, Str.		+	+
Vigna sinensis Savi				
'BlackEye'	Mo		+	+

Table 2. continued

Host plant	Symptoms	Virus recovered from[b]	
		Inoculated leaves	Uninoculated leaves
Non-legumes			
Antirrhinum majus L.	Mo	+	+
Chenopodium amaranticolor Coste & Reyn.	LL, Mo (2F)	+	+
C. hybridum L.	LL, Mo	+	+
C. quinoa Willd.	LL, Mo	+	+
Cucumis sativus L. 'National Pickling'	–	+	–
Gomphrena globosa L.	LL, Mo (2G)	+	+
Spinacia oleracea L.	Mo	+	+

---

[a] LL, local lesions; N, Necrosis; Mo, mottle or mosaic; Str, streak; –, No symptoms.

[b] As checked by the infectivity test on *C. amaranticolor*.

[c] The number of plate which illustrates the symptoms listed.

3.42 Proof of absence of WCMV in the necrotic tissues of vetch

Solid necrotic lesions were developed when the leaves of broad bean (V. faba L. 'Broad Windsor') were inoculated with the original sap sample from vetch showing severe necrosis (Plate 2B). Since WCMV was reported to produce similar lesions on broad beans (Pratt, 1961), it was suspected that WCMV was present in the original sample. The following experiments were performed to determine the presence or absence of WCMV.

1) Primary leaves of cowpea (V. sinensis Savi 'Black Eye') were inoculated with sap from the original vetch sample and attempts were made to determine the presence of WCMV in the newly emerged trifoliate leaves, according to the method of Pratt (1961). In this procedure, sap from infected cowpea incited solid local lesions and later systemic mosaic in broad bean, local and systemic mottle in C. amaranticolor and systemic mosaic in snapdragon (A. majus) (Table 2). The infection of the latter two hosts was indicative of the presence of CYMV.

2) To confirm that the local lesions produced on broad bean were incited by CYMV and not by WCMV, the sap of the infected snapdragon was back-inoculated on broad bean. This resulted in solid local necrotic lesions identical with those observed previously. Furthermore, precipitin-ring tests of sap from the systemically infected leaves of broad bean and snapdragon, carried out against

WCMV antiserum gave very low titres (1/8) in comparison with those of tests against CYMV antisera, which gave much higher titres (1/512 - 1/1024). More conclusive evidence for the absence of WCMV was obtained by electron microscopy as described in detail below.

3.43 Insect transmission

Pea plants separately inoculated by the two aphid species did not develop any disease symptoms. Moreover, the crude sap of the inoculated plants, checked for virus infectivity on C. amaranticolor, failed to produce local lesions, indicating no transmission under the conditions described.

3.44 In vitro properties

The following in vitro properties of the isolate were determined in crude pea sap. DEP: the sap was infectious at 10^{-5} dilution but not at 10^{-6}; TIP: the sap was infectious after being heated for 10 minutes at 60°C but not at 65°C; longevity: the sap was infectious after six months but not after seven months.

3.45 Electron microscopy

Electron microscopy of the negatively stained dip preparations from infected pea leaves revealed slightly flexuous rods of uniform width. The preparation contained individual particles which showed no evidence of end-to-end aggregation (Plate 7A). Figure 2 shows the results of a random measurement of 450 particles made on the electron micrographs. The major maximum length obtained from histograms of 25 nm size groupings, was found to be at 500-525 nm. The normal length was calculated as 511 nm (when average diameter of 60 latex spheres was 315 nm).

3.46 Cytological studies of virus inclusions

Several types of virus inclusions observed in the cytoplasm of infected epidermal cells are described below. Identical inclusions were seen in all hosts studied and were absent in the comparable healthy controls.

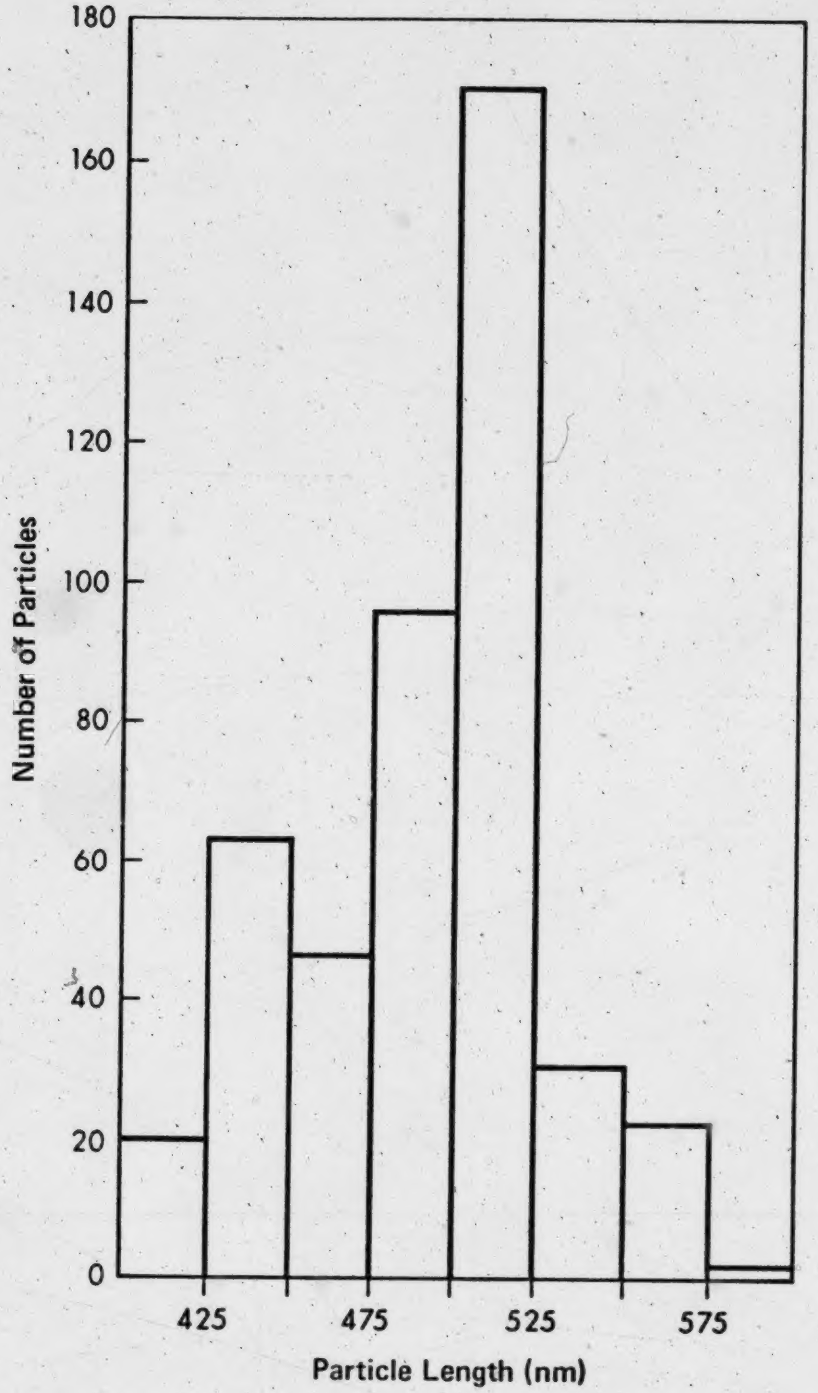
A. Light microscopy

Amorphous inclusions: These were the most common type of inclusions observed at one and two weeks after inoculation. They appeared as large aggregates mostly deposited in the corners or periphery of the cells, (Plate 3A and B) sometimes surrounded by dark bodies. Wide variation in size was noted in the inclusions both between hosts and between cells of the same host.

Banded inclusions: Parallel bands of variable width were sometimes observed extending longitudinally from one end of the cell to the other. These were filled with dense, granular material (Plate 3C).

Crystalline inclusions: These were seen only in epidermal cells sampled two weeks after inoculation, where they were not as common

FIGURE 2. Length distribution of virus particles from leaf-dip preparations of pea infected by the vetch isolate of clover yellow mosaic virus.


180
160
140
120
100
80
60
40
20
0
Number of Particles
425
475
525
575
Particle Length (nm)

as the amorphous and banded inclusions. Two kinds of crystalline bodies were observed. The first appeared as a hexagonal plate of variable size containing dark bodies, which were more conspicuous when stained with iodine-potassium iodide. The second kind was seen as a somewhat smaller, refractile, hexagonal crystal, measuring 2-4 µm in size (Plate 3D).

B. Immunofluorescence microscopy

Amorphous inclusions appeared as diffuse masses of yellowish green fluorescence mostly in the corners or periphery of infected epidermal cells (Plate 3E and F). Parallel fluorescent bands were also found in which the fluorescence was restricted to the edges of the bands.

C. Electron microscopy

To determine the structural nature and intracellular location of virus inclusions, thin sections of the infected vetch tissue were observed with the electron microscope. Plate 4A and B represents the general view of the infected leaf tissue sectioned along two different planes. Large aggregates of virus particles were observed towards the periphery of the infected cells (Plate 5A and B). These were similar to the amorphous aggregates observed in the light microscope. Sometimes these appeared as long strips tightly packed with virus particles (Plate 5C). The arrangements of virus particles appeared to be either spiral or longitudinal depending on the plane of sectioning (Plate 6). The tissue from uninoculated plants did not contain these aggregates.

3.47 Purification

The final preparation of the vetch isolate of CYMV was colourless when purified by the method described (page 32). The electron microscopic observation of a purified preparation after negative staining revealed that a majority of the virus particles occurred in end-to-end-aggregation with a few single particles (Plate 7B). The virus yield was 0.6-0.9 mg per gm of the tissue. An absorbance value of 2.5 was obtained at 260 nm when 1 mg of the virus was present in 1 ml of 0.01 M phosphate buffer, pH 7.0.

A. Sucrose density gradient centrifugation

A light-scattering zone was located 8-12 mm below the tube meniscus after density gradient centrifugation. Spectrophotometric data indicated the existence of two UV-absorbing peaks, a minor one immediately below the tube meniscus and a major one at a position corresponding to the light scattering zone. The second peak had a gradual 'shoulder' towards its base (Figure 3). Electron microscopic observations of the fractions from this peak revealed flexuous rod particles. A measurement of 143 particles gave a normal length of 516 nm. The fractions from the 'shoulder' had relatively more aggregated particles.

Infectivity was not associated with the fractions recovered from the first peak, and intact virus particles were not observed when fractions from the peak were examined in the electron microscope. However, the UV absorption spectrum for this peak resembled that of the visible zone containing virus. Hence, the material from the peak is considered to be degraded virus.

B. Ultraviolet absorption

The infective fraction (fraction 7), obtained after sucrose density gradient centrifugation, has an absorption spectrum characteristic of a nucleoprotein (Figure 4). It had a peak at 265 nm with a minimum at 245 nm. The absorption ratios at 260 nm and 280 nm for three infective fractions (6, 7 and 8) were 1.14, 1.14 and 1.15 respectively. The maximum:minimum ratio at 265 nm and 245 nm was 1.25.

C. Cesium chloride centrifugation

The virus was located as a distinct visible zone in the gradient tubes of cesium chloride (Plate 7C). The fractions obtained from this zone had a UV absorption spectrum identical to that obtained from sucrose gradient (Figure 4).

3.48 Analytical ultracentrifugation

A single distinct peak representing the virus was detected with a sedimentation ($S_{20,w}$) value in the range of 132-135 S,

FIGURE 3. Absorbance and corresponding infectivity of fractions, determined after sucrose density gradient centrifugation of the vetch isolate of clover yellow mosaic virus. Infectivity was associated with the main peak, determined by assaying on Chenopodium amaranticolor, six half leaves for each fraction. Arrow indicates a gradual 'shoulder' beneath the main peak.

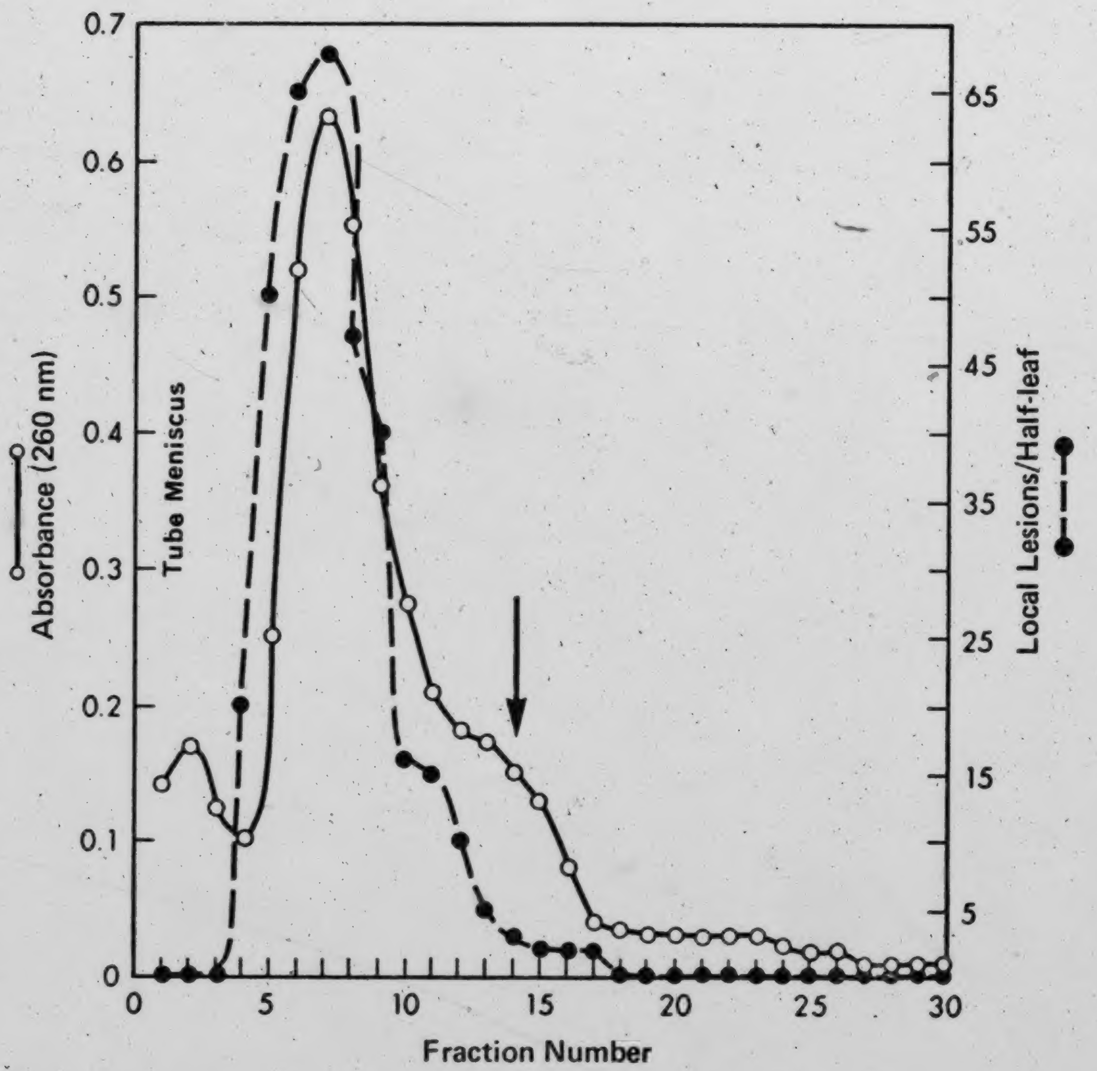
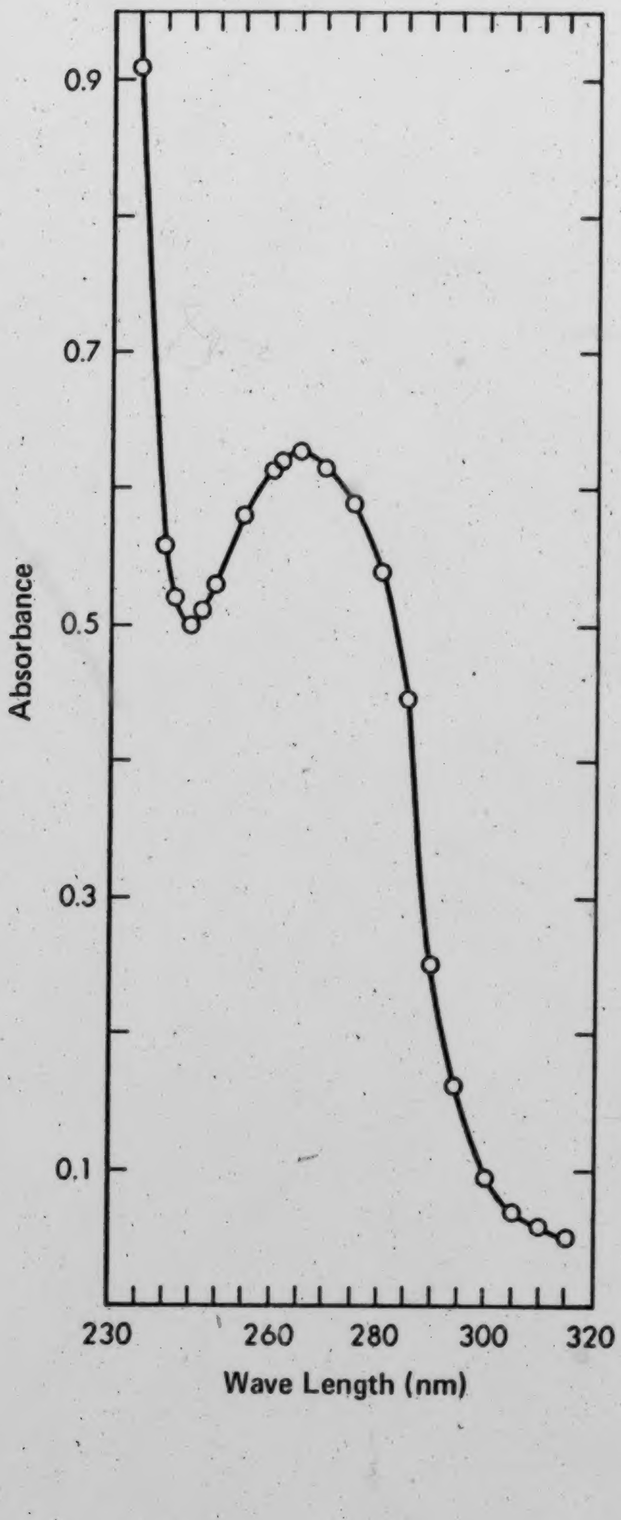

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (260 nm)
Tube Meniscus
65
55
45
35
25
15
5
Local Lesions/Half-leaf
0
5
10
15
20
25
30
Fraction Number

FIGURE 4. UV absorption spectrum of an infective fraction (fraction 7) of the purified vetch isolate of clover yellow mosaic virus, obtained after sucrose density gradient centrifugation. The spectrum was determined after dialysis of the fraction with 0.005 M phosphate buffer, pH 7.0.


0.9
0.7
0.5
0.3
0.1
Absorbance
230
260
280
300
320
Wave Length (nm)

using purified samples not subjected to sucrose density gradient centrifugation. The fraction obtained after sucrose density gradient centrifugation also sedimented as a single peak, but the sedimentation values were in the range of 145-151 S.

3.49 Serology

A. Production of homologous antiserum

The time-course study of antibody production, carried out with a single rabbit, indicated a linear increase in the antibody up to five weeks, after which the rate decreased (Figure 5), and showed a final antibody titre of 1/4096 against the vetch isolate. When antisera from seven rabbits were tested, the titres obtained ranged from 1/2048 to 1/4096. The precipitate obtained in precipitin-ring tests was of the flocculent gelatinous type described by Pratt (1961).

B. Serological relationships

The serological relationship of the vetch isolate with selected members of the potex group of viruses is presented in Table 3. The results indicated that the vetch isolate was closely related to CYMV and distantly related to WCMV and PVX. No relationship, however, was observed with cactus virus X.

C. Cross-absorption

The cross-absorption tests between the vetch isolate and

FIGURE 5. Time-course of the antibody produced against the vetch isolate of clover yellow mosaic virus in a San Juan rabbit.

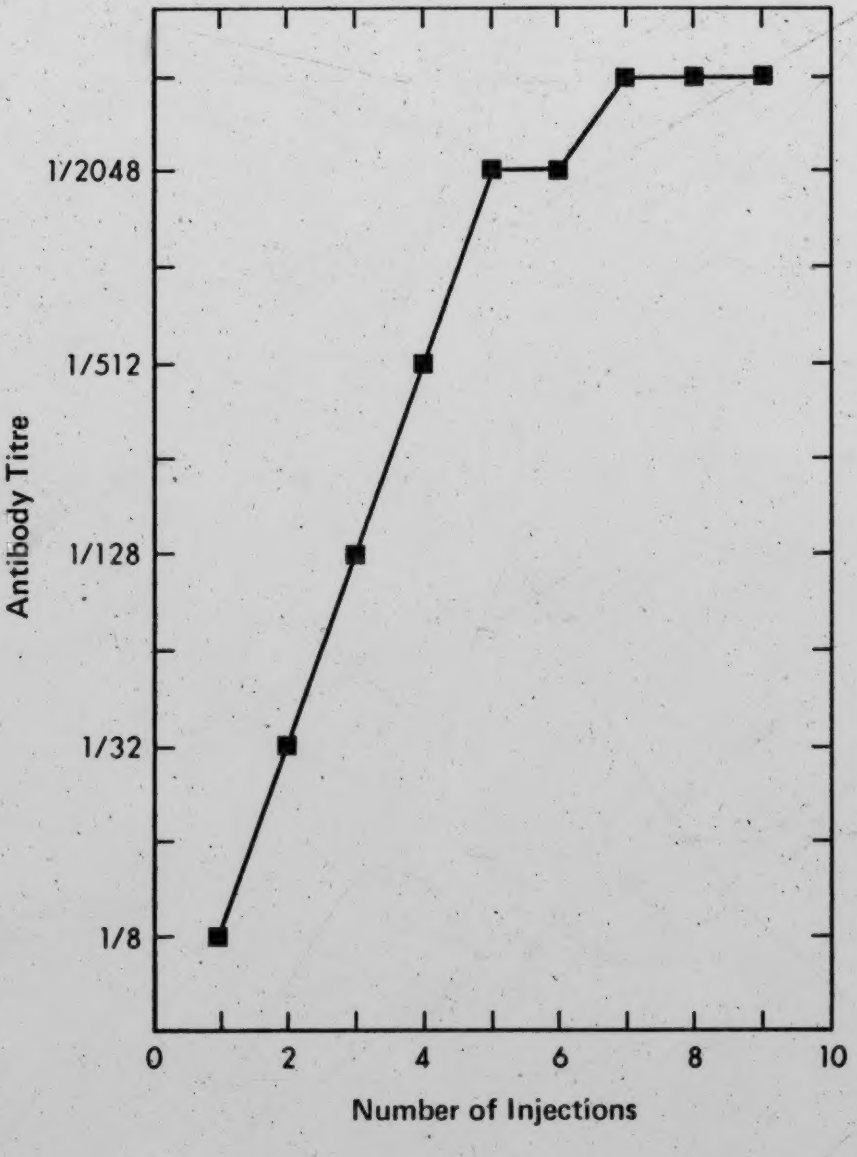

Antibody Titre
1/2048
1/512
1/128
1/32
1/8
0
2
4
6
8
10
Number of Injections

Table 3. Serological relationship as determined by the precipitin-ring tests between the vetch isolate of clover yellow mosaic virus and selected member viruses of the potex group

Antigen		Antisera						
Purified or in sap	Dilution	CYMV				WCMV	PVX	CaVX
		Vetch isolate	CYMV-S	PVAS-56	PVAS-67	PVAS-36	PV-54	PVAS-79
Vetch isolate[a]	–	1/2048[d]	1/1024	1/128	1/512	1/8	1/8	–[e]
PVX[a]	–	1/8	1/4	–	1/4	1/8	1/512	1/8
Vetch isolate[b]	1/8	1/2048	1/512	1/128	1/256	1/4	1/4	–
	1/16	1/2048	1/256	1/128	1/128	–	–	–
WCMV[c]	1/8	1/8	1/8	1/4	1/8	1/64	1/8	–
	1/16	–	–	–	–	1/32	–	–
Healthy pea sap	1/8	–	–	–	–	–	–	–
	1/16	–	–	–	–	–	–	–
Healthy bean sap	1/8	–	–	–	–	–	–	–
	1/16	–	–	–	–	–	–	–

[a] Purified virus preparation at 0.2 mg per ml.

[b] Infected Alaska pea leaf sap.

[c] Infected Bountiful bean leaf sap.

[d] Antibody titre.

[e] No precipitate.

Table 4. The results of precipitin-ring tests before and after cross-absorption between the vetch isolate and the S isolate of clover yellow mosaic virus

	Before absorption		After absorption	
Antiserum[a]	Homologous antigen	Heterologous antigen	Antigen Vetch	Antigen CYMV-S
CYMV-S	1/2048[b]	1/1024	0	1/64
Vetch	1/2048	1/1024	1/128	0

---

[a] The CYMV-S antiserum was absorbed with the vetch isolate antigen and vice versa. Purified virus preparations were used as antigen at 0.3 mg/ml.

[b] Antibody titre.

CYMV-S indicated that neither of the isolates could completely cross-absorb the antibody of the other, indicating a serological distinctness between the isolates (Table 4).

3.60 DISCUSSION

Natural infections of legumes such as clover, alfalfa and pea with CYMV, either alone or in combination with other legume viruses, have been reported (Pratt, 1961; Ford and Bagget, 1965). The results presented in this thesis report, for the first time, the natural occurrence of CYMV causing severe necrosis on field vetch. Since vetch is grown as a perennial forage crop in Alberta, the infected plants may constitute an important potential source of CYMV inoculum for other annual and perennial legumes in the province. No evidence, however, was obtained to suggest the possible existence of WCMV as a complex with CYMV in the vetch necrosis. The local, solid necrotic lesions produced after infection with the vetch isolate on broad beans may be attributed to differences in the varietal reaction or virus isolate used.

Although the vetch isolate has a host range similar to that of the CYMV isolates previously reported (Pratt, 1961), it differs in its symptomatology on vetch, broad bean and cucumber. The normal particle length reported for the vetch isolate in the present investigation is comparable to those reported previously for CYMV from other laboratories (Pratt and Reichmann, 1961; Agrawal et al., 1962b; Bercks and Brandes, 1963). However, Welsh et al. (1973) reported a modal length of 590± 10 nm for the apple isolate.

In insect transmission studies, the two aphid species A. pisum and M. persicae failed to transmit the virus under the described conditions. Pratt (1961) also failed to transmit CYMV by A. pisum, although the acquisition and inoculation feeding periods used were much longer than those used in the present study. It should be pointed out that more experiments are needed using different species of aphids and shorter acquisition feeding periods before any final conclusions are made.

The light and electron microscope studies of the infected tissue revealed three types of inclusions, characteristic of CYMV (Purcifull, et al., 1966; Christie, 1967). The crystalline inclusions detected by light microscopy in this investigation, however, have not previously been described. Their absence in the epidermal cells sampled one week after inoculation suggests that, unlike amorphous inclusions, they were formed in the later stages of infection. The internal structure of banded inclusions is unclear. It is interesting to note, however, that the presence of virus aggregates resembling the banded structures was observed in the thin sections of the infected vetch tissue (Plate 5C).

Antigenicity of the non-crystaline inclusion bodies formed after infection with CYMV was reported by Schlegel and Delisle (1971). Other reports by Nagaraj and Black (1961) and Sinha and Black (1962,

1963) also demonstrated viral antigenicity in situ with other virus infections. However, the method employed to demonstrate the antigenicity of CYMV in the present work is simple and efficient for two reasons. Firstly, there is no need to section the material since epidermal cells are thin-walled and allow the fluorescent antibody to penetrate them. Secondly, the fluorescence can be observed with the light microscope.

Fluorescence was observed throughout the body of amorphous inclusions, whereas parallel bands fluoresced only at the edges. The reason for such a difference can be interpreted in two ways. Firstly, the parallel bands may represent tightly packed virus particles which might have restricted the diffusion of the antibody in the inclusion. Secondly, they may be serologically active at the periphery only, as described for CYMV inclusions (Schlegel and Delisle, 1971).

The PEG method used for the preparation of the highly purified vetch isolate has several advantages over some other methods. The use of organic solvents, harmful to virus in certain cases, was unnecessary and avoided in this investigation, and the method requires only two differential cycles of centrifugation to obtain highly purified preparations. High virus infectivity was retained throughout purification. However, judging from the sucrose density gradient sedimentation patterns, this method resulted in as much viral aggregation as was obtained by the other methods used in this investigation for comparison (Pratt, 1961; Agrawal, et al., 1962b).

The sedimentation value of 132-135 S for the vetch isolate is higher than those reported for other isolates of CYMV (121 S, Pratt and Reichmann, 1961; 122 S, Welsh et al., 1973) inspite of the use of identical buffer systems. The reason for the higher sedimentation rate is unclear. It is possible that certain amount of aggregation in the purified preparation might have contributed to the higher sedimentation rate. Similarly, a sedimentation value of 145-151 S obtained with CYMV prepared by sucrose gradient centrifugation may reflect a similar situation.

Based on the similarities in the host range, particle morphology, _in vitro_ properties, inclusion bodies, sedimentation properties and serology, the vetch isolate is identified as CYMV. Furthermore, this isolate can be distinguished from the other Canadian isolate tested, CYMV-S by the severity of symptoms on vetch and by serological differences.

CHAPTER 4

FACTORS AFFECTING THE INFECTIVITY ASSAY OF CLOVER YELLOW MOSAIC VIRUS IN CHENOPODIUM AMARANTICOLOR

4.10 INTRODUCTION

C. amaranticolor, G. globosa, P. aureus (Pratt, 1961) and C. quinoa (Welsh et al., 1973) have been reported to produce local lesions when infected with CYMV. C. quinoa (Welsh et al., 1973) has been used both as an indicator and as a quantitative assay host for an apple isolate of CYMV. Ford (1973) and Smith and Schlegel (1964) used G. globosa for infectivity assay of CYMV. In all these studies, and in the quantitative assays especially, conclusions were made apparently without detailed knowledge of the various factors that could influence the assay results. Since such knowledge is essential to the development of a reliable method of infectivity assay, this study was undertaken for CYMV using C. amaranticolor as a local lesion host.

4.20 MATERIALS AND METHODS

4.21 Assay plant

The seeds of C. amaranticolor were washed in running tap water for 3-4 hours and germinated at 25°C on moist filter paper for three days. Uniformly germinating seeds were transferred to plastic trays containing a soil mixture of loam, sand and peat in a 3:2:1 ratio. Fifteen days later, individual seedlings were transplanted to autoclaved 12 cm clay pots. The assay plants were grown in a greenhouse under the conditions described in Chapter 3 (page 28). Plants, about two months old, bearing 14 leaves, excluding the bottom two abscised leaves and top two pigmented but unexpanded leaves, were used. The leaves were numbered in sequence from bottom to top.

4.22 Inoculum

The sap inoculum was obtained from pea plants showing severe mosaic symptoms three weeks after inoculation. To minimize variability, a single batch of infected pea plants grown under identical conditions was used as an inoculum source. The individual leaves from 20 plants were detached, mixed well, divided into 0.5 gm individual samples, and kept frozen at -20°C until they were used for the tests. Inoculum was prepared by grinding 0.5 gm of frozen tissue in 4.5 ml of 0.01 M phosphate buffer, pH 7.0, with a mortar and pestle at room temperature. The sap was strained through a double layer of cheesecloth and diluted to 10^{-4} with the same buffer. Preliminary tests with different dilutions indicated that a 10^{-4} dilution of the sap usually gave a lesion number which was easily countable. Hence, in all experiments, the standard dilution of 10^{-4} was used. Phosphate buffer solutions were prepared by mixing equimolar solutions of $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4. The pH of the buffer solution was determined with a glass electrode.

The four leaves at positions 8, 9, 10 and 11 of two-month-old C. amaranticolor plants were selected for inoculation for reasons explained in the results section, and half leaves or whole leaves were used as an inoculation unit, depending on their suitability for a given experiment. Where the half-leaf technique was employed the treatments were arranged in a balanced pattern. Usually 12 or 16 replicates were used in testing a sample of inoculum. All leaves of the plants chosen for inoculation were uniformly dusted with 22 µm (600-mesh) Carborundum, and inoculated by stroking with a cotton swab dipped in inoculum. The inoculated leaves were rinsed with distilled water immediately after inoculation. Unless otherwise stated, the inoculated plants were incubated in growth chambers with standard conditions of temperature 21°C ± 2°C, 16 hours light period at a light intensity of 10,760 lux and at about 70% relative humidity.

To obtain dilution curves, virus purified according to the method described in Chapter 3 (page 32) was used.

The lesion counts were processed with a computer (Model Amdhal 470) by using one-way analysis of an SPSS (Statistical Package for the Social Sciences) program (Kim and Kohout, 1975).

Duncan's multiple range test (Duncan, 1965) was employed to determine the significance between the treatments. The lesion counts and the results of Duncan's multiple range tests are included in the appendix.

4.30 RESULTS

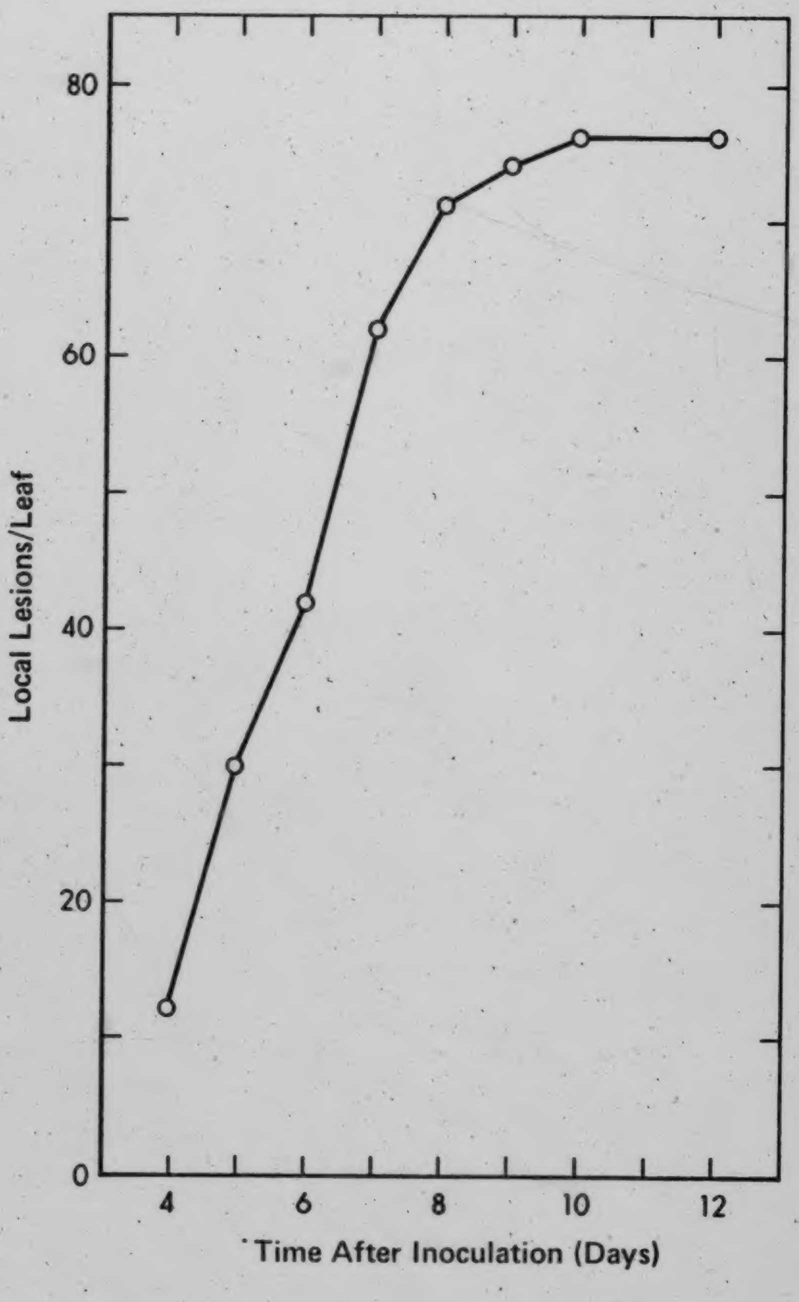
4.31 Time-course study of increase in local lesion number after inoculation

To determine the optimal time after inoculation for counting the lesions, the time-course of increases in the local lesion number was investigated over a period of 12 days after inoculation at 21°C. The lesions which first appeared four days after inoculation, and were minute and water-soaked in appearance. With increasing time after inoculation they increased in size and number, and became discrete with chlorotic margins (Plate 2F). This increase was linear up to eight days after inoculation, started to level off thereafter up to 12 days and the rate of lesion increase during this period was very small (Figure 6). Hence, the lesions were counted nine to ten days after inoculation in all experiments.

4.32 Age of the plant and position of the leaf

Preliminary tests with younger plants, three to six weeks old, indicated that the virus produced many pinpoint lesions which were difficult to count. Hence, two-month-old plants were used

FIGURE 6. Time-course of increase in local lesion number in _Chenopodium_ _amaranticolor_ leaves inoculated with clover yellow mosaic virus. Each point represents an average lesion number of 16 leaves.


80
60
40
20
0
Local Lesions/Leaf
4
6
8
10
12
Time After Inoculation (Days)

at a growth stage when 14 inoculable leaves were present. Plants with fleshy, thick and green leaves gave more discrete lesions upon inoculation than those with thin and yellowish leaves. Therefore the plants were carefully selected for uniformity prior to inoculation, preferably in the summer, when plants with the desired texture were more readily encountered than in the fall.

Table 5 shows the results of a test in which all 14 leaves were inoculated and the average number of lesions at each leaf position was counted. The lower leaves at positions 3, 4, 5 and 6 showed non-discrete, hazy lesions which were difficult to count. Among the leaves from positions 7-14, the leaf size, lesion size and number were more uniform at positions 8-11 than those at other positions. Hence, the leaves at the positions 8-11 were used in subsequent infectivity assays.

4.33 Post-inoculation temperature

Groups of plants with a total number of 12 or 16 inoculable leaves were inoculated and placed in controlled growth cabinets (Model E 30, Percival Inc., Boone, Iowa, U.S.A.) individually set at different temperatures 17°, 21°, 25° and 29°C, with a light period of 16 hours and a light intensity of 10,760 lux. The results are shown in Table 6. Daily lesion counts were made to determine the optimal time for counting at each temperature (Figure 7). As the temperature was raised, there was an accompanying decrease

Table 5. Number of local lesions incited by clover yellow mosaic virus in the leaves of *Chenopodium amaranticolor* at different leaf positions

Leaf position	Number of lesions
14	46[a]
13	63
12	84
11	77[b]
10	75
9	70
8	68
7	48
6	–
5	–
4	–
3	–
2	–
1	–

[a] Average number of lesions for 10 leaves obtained at the same leaf position on different plants.

[b] Bracket denotes the leaf positions suitable for infectivity assay at which uniform leaf size, lesion size and number are obtainable.

in the incubation period, the shortest being at 29°C. At this temperature, the increase in local lesion number was very rapid up to five days, after which the lesions coalesced, and the resulting large chlorotic patches made counting impossible. At 21°C, the increase was slower and the number continued to rise even up to 14 days. The results suggest that the temperature range of 21°-25°C is adequate for CYMV infectivity assay.

4.34 Dark treatment

The effect of dark treatment on the susceptibility of *C. amaranticolor* was determined at various time intervals. The plants were given pre-inoculation dark treatments of 24, 48 and 72 hours, timed so that, together with plants given no dark treatment (zero hour serving as controls), all plants could be inoculated at the same time. Twelve leaves were used for each treatment. The average number of lesions for the two experiments are as follows: zero hour: 73.8 ± 4.8, 67.0 ± 6.8; 24 hours: 80.1 ± 3.4, 66.7 ± 5.9; 48 hours: 69.3 ± 4.5, 65.3 ± 5.8; 72 hours: 69.8 ± 6.6, 63.4 ± 6.1. There were no significant differences between control and treatment values, suggesting that the dark treatment did not affect the susceptibility of the host (Table 18, appendix).

4.35 Light intensity

Groups of three or four plants were incubated in growth

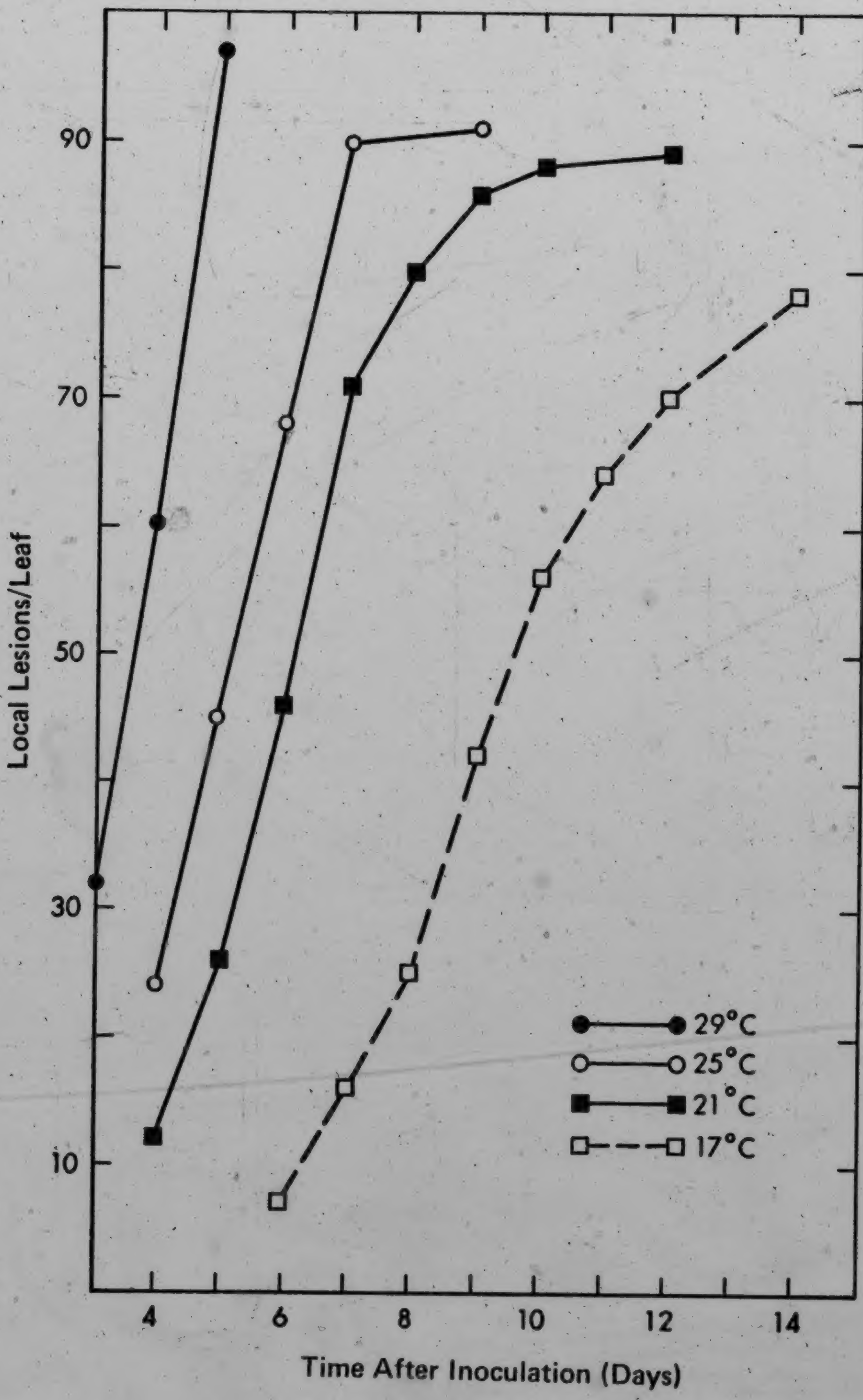
Table 6. Effect of post-inoculation temperature on the number of local lesions incited by clover yellow mosaic virus in *Chenopodium amaranticolor* leaves

Temperature (°C)	Experiments[a] I	II	III
17	69.8[b] ± 3.0	35.9 ± 3.0	77.0 ± 5.9
21	85.6 ± 3.6	60.7 ± 4.9	101.4 ± 6.0
25	88.9 ± 5.0	62.6 ± 4.4	103.0 ± 4.7
29	97.4 ± 6.9	77.9 ± 6.0	119.5 ± 5.2

[a] Lesions were counted 12 days after inoculation at 17°C; 9 days after inoculation at 21°C; 7 days after inoculation at 25°C; 5 days after inoculation at 29°C.

[b] Average number of lesions for 12 or 16 leaves.

FIGURE 7. A comparison of local lesion numbers obtained in Chenopodium amaranticolor leaves incubated at 29°, 25°, 21° and 17°C respectively, after inoculation with clover yellow mosaic virus.


Local Lesions/Leaf
90
70
50
30
10
4
6
8
10
12
14
Time After Inoculation (Days)
●—● 29°C
○—○ 25°C
■—■ 21°C
□- - -□ 17°C

cabinets equipped with fluorescent lights providing light intensities of 6456, 10760, 16140 and 21520 lux (600, 1000,1500, 2000 ft. candles respectively). The results of three experiments are presented in Table 7. Since the three experiments were carried out separately, no comparison could be made between the experiments. However, the numbers of lesions in all the experiments at 21,520 lux were significantly lower than those obtained at the other three light intensities tested (Table 22, appendix). Hence, a light intensity range between 6,456 - 10,760 lux was used in subsequent infectivity assays. At higher light intensities, the lesions were larger in size and surrounded by reddish brown halos.

4.36 Molarity of phosphate buffer

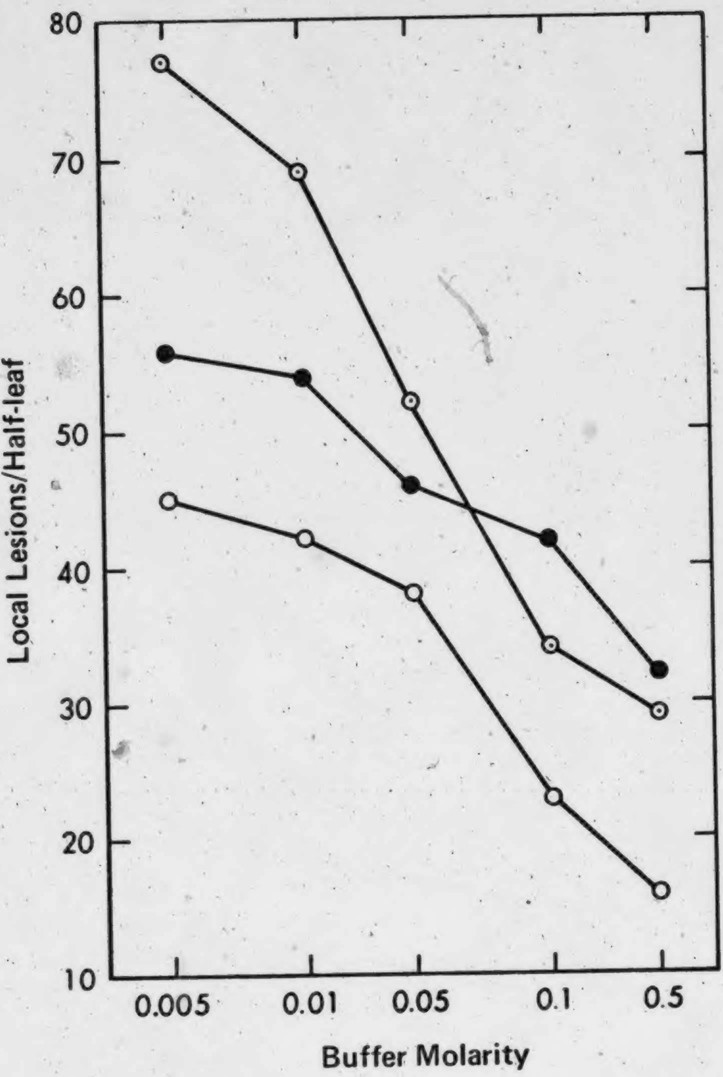
CYMV extracts obtained in phosphate buffers, pH 7.0 of different molarities were used as inocula. Twelve half-leaves were used for each concentration. The results showed increases in lesion number with decreased molarity (Figure 8). The molarity range from 0.005 M to 0.05 M, in which the lesion numbers were generally the highest, is the one most suitable for infectivity assay as the lesion numbers were not significantly different from each other in two experiments out of three (Table 26, appendix). When sap was extracted in distilled water (pH adjusted to 7.0) the lesion number obtained was lower than the numbers obtained from the above range of molar concentrations of buffer

Table 7. Effect of light intensity on the number of local lesions in *Chenopodium amaranticolor* leaves inoculated with clover yellow mosaic virus

Light intensity (lux)	Experiments[a]		
	I	II	III
6,456	53.3 ± 2.9	44.3 ± 4.3	35.3 ± 5.6
10,760	65.6 ± 3.7	51.9 ± 4.7	42.6 ± 3.8
16,140	36.4 ± 2.4	37.0 ± 4.0	32.5 ± 1.4
21,520	17.5 ± 1.8	11.7 ± 1.6	15.7 ± 1.6

[a] Average number of local lesions in 12 (Expt. II) and 16 leaves (Expt. I and III). Temperature of the growth chamber was adjusted to 21°C. At higher light intensities, temperature at leaf levels often exceeded the set temperatures by a few degrees.

FIGURE 8. Effect of different molar concentrations of phosphate buffer, pH 7.0, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions in *Chenopodium amaranticolor* leaves. The results of three experiments are shown (o——o, Exp. I; ●——●, Exp. II; ⊙——⊙, Exp. III).


80
70
60
50
40
30
20
10
Local Lesions/Half-leaf
0.005
0.01
0.05
0.1
0.5
Buffer Molarity

(Tables 23, 24 and 25, appendix).

4.37 pH of phosphate buffer

CYMV was extracted in 0.01 M phosphate buffer at the range of pH values 5.5 - 9.0. Inocula were each divided into two batches and separately applied to two groups of C. amaranticolor plants. Twelve or 16 half-leaves were used for testing each sample. The local lesion numbers obtained in the pH range 6.5 - 8.0 were not significantly different from each other (Table 29, appendix). Hence, this range was considered optimal for assays of CYMV infectivity (Figure 9).

4.38 Dilution curve

The initial concentration of the purified virus preparation was determined by spectrophotometry and the virus inocula with final concentrations 1, 2, 4 and 6 µg per ml were subsequently prepared in 0.01 M phosphate buffer, pH 7.0. Sixteen half-leaves were used for testing each concentration. The results show a nearly linear relationship between the local lesion number and the amount of CYMV applied within the range of dilutions tested (Figure 10).

4.40 DISCUSSION

The factors that influence the local lesion number incited in some species of Chenopodium by certain plant viruses have been investigated by several workers (Rochow, 1959; Hagedorn et al.,

FIGURE 9. Effect of pH of 0.01 M phosphate buffer, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions in *Chenopodium amaranticolor* leaves. The results of two experiments using the same batch of inoculum are shown (●——●, Exp. I; ○——○, Exp. II).

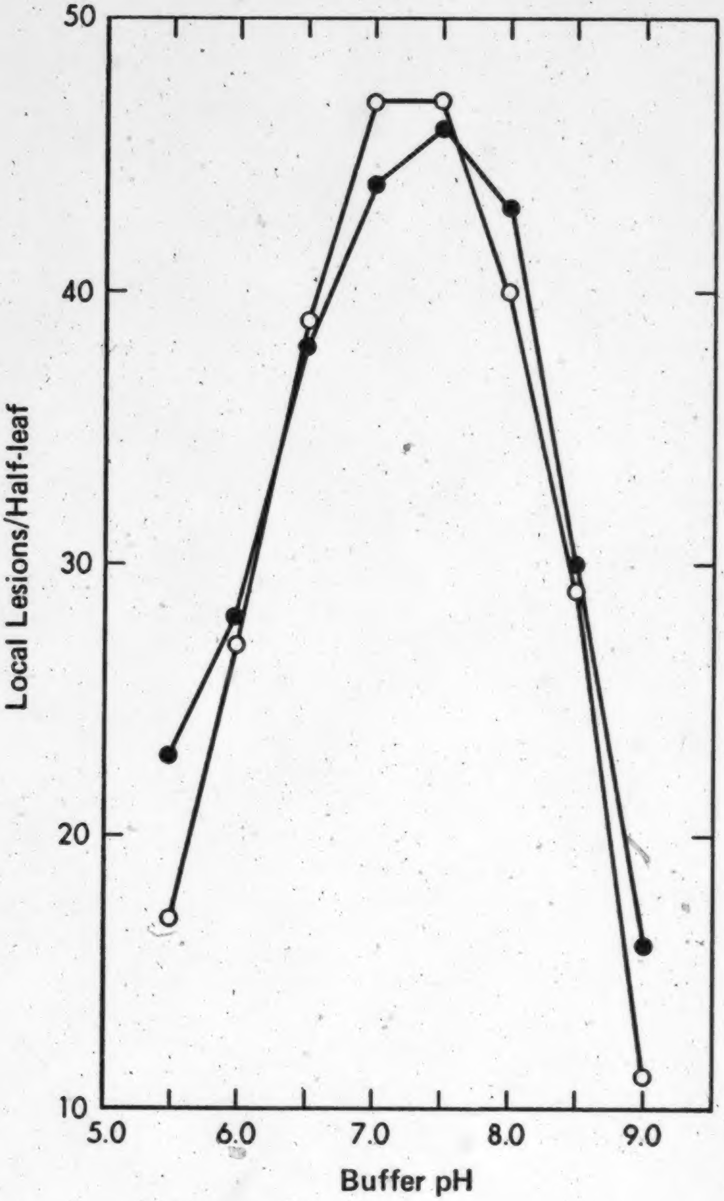
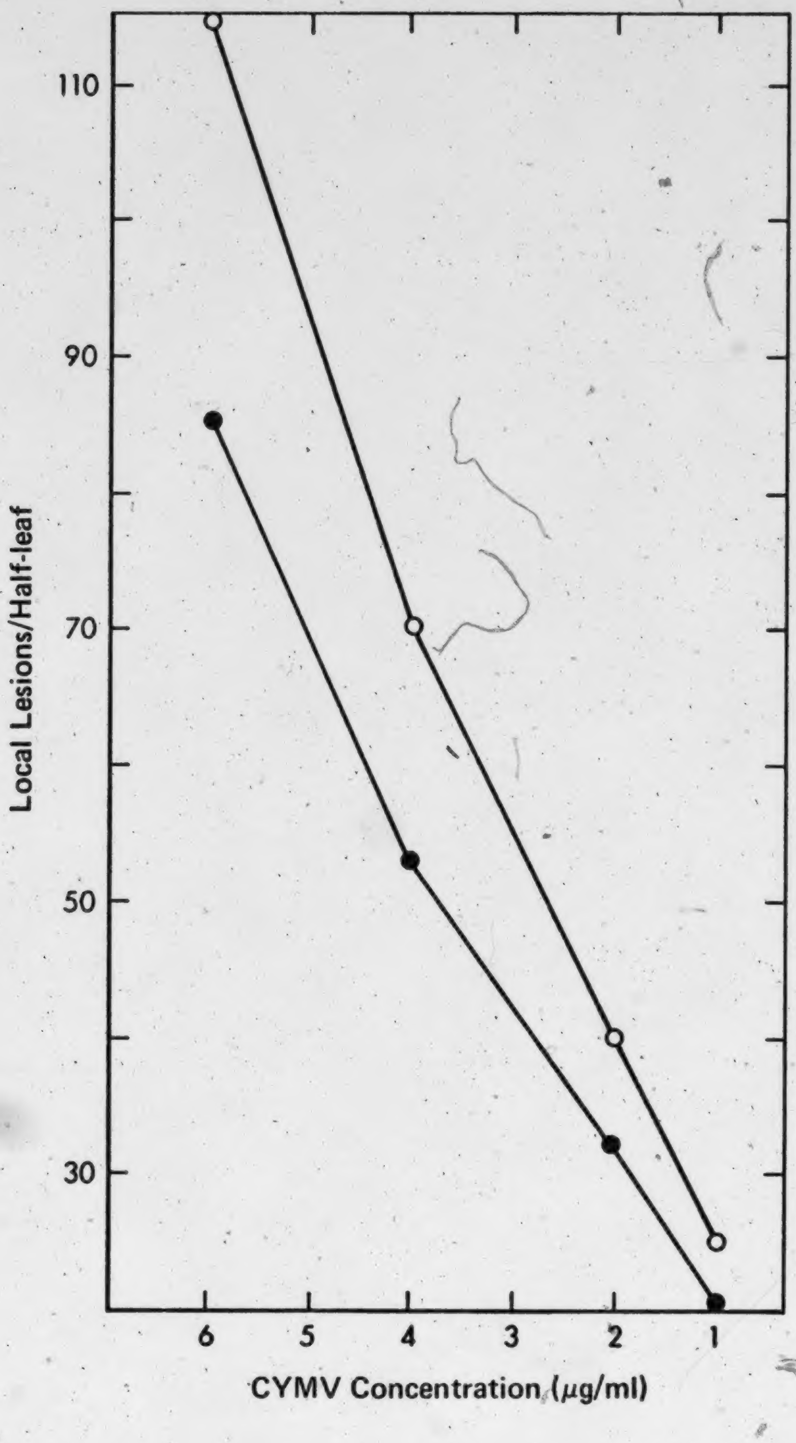

50
40
30
20
10
5.0
6.0
7.0
8.0
9.0
Local Lesions/Half-leaf
Buffer pH

FIGURE 10 Dilution curves obtained with two purified preparations of clover yellow mosaic virus (●——●, Exp. I; o——o, Exp. II).


Local Lesions/Half-leaf
110
90
70
50
30
6
5
4
3
2
1
CYMV Concentration (μg/ml)

1964; Rosenkranz and Hagedorn, 1964; Delgado-Sanchez and Grogan, 1966; Foster, 1967, 1972; Singh, 1969; Hiruki, 1975). In the present investigation, C. amaranticolor has several advantages as a local lesion host for CYMV. Firstly, it is very sensitive to infection by CYMV; even at a sap dilution of 10^{-4}, the number of local lesions produced is substantially high. Secondly, its leaves are much larger than those of other local lesion hosts, and hence are more suitable for assaying. Thirdly, it is susceptible to infection throughout its growth period, although older plants are preferable to younger ones. Fourthly, it is not susceptible to WCMV, hence contamination with this virus can be avoided (Pratt, 1961).

There have been several reports indicating that plants grown under reduced lighting (Bawden and Roberts, 1947, 1948) or shaded (Ross, 1953) or dark-treated before inoculation (Rosenkranz and Hagedorn, 1964; Trujillo and Saettler, 1972; Hiruki, 1975) can produce substantially increased number of lesions. Preinoculation dark treatment in particular is known to be effective in increasing host susceptibility, but only under certain conditions. Thus Matthews (1953b) has reported that this effect to some extent was obscured by varying periods of light and darkness after inoculation, and Wiltshire (1956) has reported greater susceptibility effects in summer than in winter. The results of the present study did not suggest any significant increase in host susceptibility by the dark treatment. While this may be attributed to the different host-virus system used, it is possible that the conditions of light and darkness after inoculation influenced the results. It will also be of interest to study the effects of different light intensities immediately after dark treatment, since it has been reported that a doubling of lesion number occurred when dark-treated leaves had been exposed to a short burst of light prior to inoculation (Helms and McIntyre, 1967a, b).

The effect of the phosphate ion in increasing the number of lesions is a well known phenomenon for certain host-virus systems. The results in this investigation confirm the previous reports (Thornberry, 1935; Yarwood, 1952, 1962). It is interesting to note, however, that higher numbers of lesions were obtained as the molarity of buffer decreased in the range tested. This may have been due to less aggregation of virus particles at lower ionic strengths, which might have made more individual virus particles available to initiate infection. However, the results of this investigation do not provide evidence to refute earlier conclusions (Thornberry, 1935; Yarwood, 1952, 1962) that the increases in the lesion number are due to the effect of the phosphate ion on the host plants.

The dilution curve with purified virus indicated a linear relationship between the lesion number and concentration of the virus up to 4 µg per ml, above which a tendency to deviate from the linear relationship was observed. From these results, a virus concentration of 4 µg per ml can be suggested as being suitable for infectivity assay with purified virus inoculum.

Although C. amaranticolor has several advantages as an effective local lesion host, careful selection of test leaves with the proper texture is an important prerequisite to obtain discrete lesions. During the fall months, light green, thin leaves gave very faint lesions which were hard to count. It may also be possible to obtain uniform plants that are suitable for inoculation for an extended period of time if plants are grown under controlled conditions.

CHAPTER 5

FACTORS AFFECTING THE INFECTION OF COWPEA MESOPHYLL PROTOPLASTS WITH CLOVER YELLOW MOSAIC VIRUS

5.10 INTRODUCTION

There are only a few reports of plant virus infection using protoplasts isolated from plant species outside the Solanaceae (Hibi et al., 1975; Ranaudin et al., 1975). With the cowpea, a species belonging to the Papilionaceae, mesophyll protoplasts have been successfully used in studies of CPMV (Beier and Bruening, 1975; Hibi et al., 1975), TMV and CMV (koike et al., 1976). Since CYMV infection of intact cowpea leaves results in a very high virus concentration (Rao, unpublished results), this host has been chosen for protoplast isolation and virus multiplication studies. In the present investigation, the various factors that affect the infection and multiplication of CYMV in cowpea mesophyll protoplasts are investigated.

5.20 MATERIALS AND METHODS

5.21 Isolation of cowpea mesophyll protoplasts

Cowpea seeds were incubated on a moist filter paper in a petriplate at 28°C for two days. The germinated seeds were planted in moist vermiculite of fine grade and were grown in a controlled growth cabinet (Model E 30, Percival Co., Boone, Iowa, U.S.A.) at an approximate relative humidity of 80%. A 14-hour light period at approximately 11,836 lux (1100 ft. candles) supplied by six fluorescent lamps (F24712/CW/HO, Sylvania Ltd., Montreal, Canada) and two 40-watt incandescent bulbs and at a temperature of $28^oC \pm 2^oC$ was alternated with a 10-hour dark period at $23^oC \pm 2^oC$.

The plants received half strength Hoagland's solution throughout the growth period. The one-step procedure of Hibi et al. (1975) was used for isolation of protoplasts. For quantitative work, the protoplasts were counted with a haemocytometer.

5.22 Inoculation

Unless otherwise stated, protoplasts were inoculated by the direct method. The purified preparation of CYMV was diluted to 10 µg per ml in 0.01 M potassium citrate buffer, pH 5.8, prepared in 0.6 M mannitol. The concentration of poly-L-ornithine (mol. wt. 150,000, Pilot Chemicals Co., Boston, Mass., U.S.A.) was adjusted to 0.8 µg per ml in citrate buffered mannitol. A 2 ml aliquot of poly-L-ornithine, mixed with an equal amount of virus solution and kept at 25^oC for 30 minutes, was used to suspend freshly sedimented protoplasts, the concentration of which was adjusted to 2.5×10^5 per ml. Final concentrations were thus 0.4 µg per ml poly-L-ornithine, 5 µg per ml virus, 2.5×10^5 per ml protoplasts and 0.01 M potassium citrate buffer, pH 5.8. The samples were shaken at 25°C for 15 minutes at 90 excursions per minute.

Where the indirect method was used, freshly sedimented protoplasts were suspended in 0.01 M citrate buffered mannitol, pH 5.8, and the concentration was adjusted to 5×10^5 per ml protoplasts. The virus-polyL-ornithine mixture, incubated as in the direct method, had a concentration of 0.8 µg per ml poly-L-ornithine and 10 µg per ml virus. This mixture was added to the protoplast suspension at a one-to-one volume ratio, so that final concentrations were the same as those in the direct method.

In the direct and indirect methods, the protoplasts were washed after inoculation at least three times in 0.6 M mannitol containing 10 mM $CaCl_2$ to remove unadsorbed virus.

5.23 Incubation

Protoplasts were incubated in 10 ml aliquots of standard incubation medium in 125 ml Erlenmeyer flasks according to Hibi et al. (1975), except that the concentration of cephaloridine was reduced to 500 µg per ml. After the incubation period, 3 ml aliquots were removed for a fluorescent antibody test and the remaining 7 ml aliquots were collected by centrifugation, the pellets being suspended in 0.6 M mannitol. The protoplasts were then washed twice , repelleted and kept at -20°C for use in the infectivity assay.

5.24 Infectivity assay

The frozen protoplasts were thawed at room temperature, suspended in 0.25 ml of 0.005 M phosphate buffer, pH 7.0, and the aliquots ground individually in glass tissue grinders (Bellco Biological Glassware, Vineland, U.S.A.). The homogenates were centrifuged at 17,000 g for 15 minutes, The supernatants were adjusted to a final volume of 0.25 ml with the same buffer. Inoculation of C. amaranticolor plants was made by the half-leaf method using eight half-leaves for each treatment. The inocula were applied in a balanced pattern.

5.25 Fluorescent antibody

A. Preparation of immunoglobulin

The immunoglobulin fraction of the antiserum was precipitated with ammonium sulfate according to Campbell et al. (1964), except that phosphate buffered saline (PBS) was used instead of borate buffered saline. After the third precipitation, the precipitate was dissolved in and dialyzed against PBS until it was free from ammonium sulfate as checked by the silver nitrate test.

B. Conjugation

After adjusting the protein concentration of the preparation to 1%,conjugation with FITC (ICN Pharmaceuticals Inc., Ohio, U.S.A.) was performed according to Otsuki and Takebe (1969). The conjugated preparation had a staining titre of 1/32 and the ratio of fluorescein to protein, determined by absorbancies at 495 nm and 280 nm, was 1.7. The preparation was stored at -20°C in small glass tubes in 1 ml lots until use.

To minimize non-specific staining, an acetone powder preparation of healthy pea leaves was mixed with the conjugated globulin at a rate of 50 mg per 2 ml for 2 hours at room temperature. The mixture was then centrifuged at 2000 g for 15 minutes to obtain the supernatant.

Normal anti-rabbit globulin, prepared as described for anti-CYMY globulin, served as control.

C. Staining

The staining method of Otsuki and Takebe (1969) was modified to minimize the undesirable effects of direct dehydration,such as distortion and crumbling of protoplasts. Protoplasts were prefixed in 3% glutaraldehyde for 1 hour and washed twice with PBS. A drop of thick suspension was placed on a glass slide previously smeared with Meyer's albumin and dried quickly by a stream of warm air. The slide was then immersed in 95% ethanol for 10 minutes and washed for 30 minutes in PBS, after which a drop of fluorescent antibody was applied to the fixed sample and incubated for 2 hours at 37°C in a water bath. The slide was then washed with two changes of PBS for 90 minutes, mounted in phosphate buffered glycerol and examined with a Leitz Ortholux I microscope fitted with a 200 W ultra high pressure mercury lamp. The exciter filters UG I (1 mm), BG 3 (1 mm) and a red suppression filter BG 38 (4 mm) were used with an eyepiece barrier filter K 490. Observations were recorded with an automatic camera using a Kodak Tri-X film.

Except for time course studies, protoplasts were harvested 48 hours after inoculation for fluorescent antibody staining. Usually, 300 protoplasts were counted to assess the percentage of protoplasts showing fluorescence in each experiment.

5.26 Scanning electron microscopy

The protoplasts were fixed in 3% paraformaldehyde-glutaraldehyde in 0.2 M Millonig's buffer, pH 7.4, for 3 hours, then post-fixed in 2% osmium tetroxide, and passed through a graded series of ethanol, followed by a concentration series of amyl acetate. They were then dried by the critical point method using liquid carbon dioxide as a transitional fluid in a Denton vacuum drier (Model DCP-1). The dried protoplasts were examined with a Cambridge Stereoscan S4 electron microscope operated at 20 kV and 15-30° tilt.

5.30 RESULTS

The one-step procedure, applied to the primary leaf tissues of cowpea, yielded approximately 1-3 x 10^6 protoplasts per gm (fresh weight) of leaf tissue. From 80 to 83% of the protoplasts were apparently intact when counted immediately after isolation and washing. Plate 8A and B shows the appearance of the protoplasts as observed with the light and the scanning microscope respectively. A majority of the epidermal protoplasts were removed by low-speed centrifugation, although sometimes from 1 to 3% of a total protoplast population were found to be of the epidermal origin. This low percentage, however, did not seem to interfere with the infection studies.

5.31 Method of inoculation

To evaluate the relative efficiencies of methods of inoculation, both the direct and indirect methods were compared. The results, representing the percentage of infected protoplasts from three experiments for the direct/indirect methods, are as follows: 28/24, 20/19, 29/31. These results indicate no significant differences between the two methods. Although either of them could be used in inoculations, the direct method was preferred in subsequent experiments for its simplicity.

5.32 Concentration of poly-L-ornithine

When protoplasts were inoculated in the absence of poly-L-ornithine, only 4-9% of them were infected. Hence, experiments were carried out to investigate the effect of poly-L-ornithine on the rate of infection. The percentage of infected protoplasts was highest at 0.6 µg per ml. At this level, however, the survival rate of protoplasts was 45%. A lower poly-L-ornithine concentration (0.4 µg per ml) gave a significantly higher percentage survival of protoplasts (Figure 11).

5.33 Pre-incubation of virus with poly-L-ornithine

The percentage of infected protoplasts was almost doubled when the virus was pre-incubated with poly-L-ornithine for 40 minutes (Table 8). However, a pre-incubation period of 10-30 minutes seems to be adequate to maintain higher protoplast survival rates.

5.34 Duration of contact of virus with protoplasts

Immediately after adding a virus-poly-L-ornithine mixture to protoplasts, the mixtures were incubated for different periods of time in a shaker bath. A brief contact of virus with protoplasts for 15 minutes gave rise to a reasonably high percentage of infection which did not change appreciably with increasing incubation periods up to 60 minutes (Table 9).

5.35 Concentration of virus inoculum

A maximum number of infected protoplasts was obtained at an

FIGURE 11 Effect of different concentrations of poly-L-ornithine on the infection and survival of cowpea mesophyll protoplasts. Protoplasts showing fluorescence were counted 48 hours after inoculation.

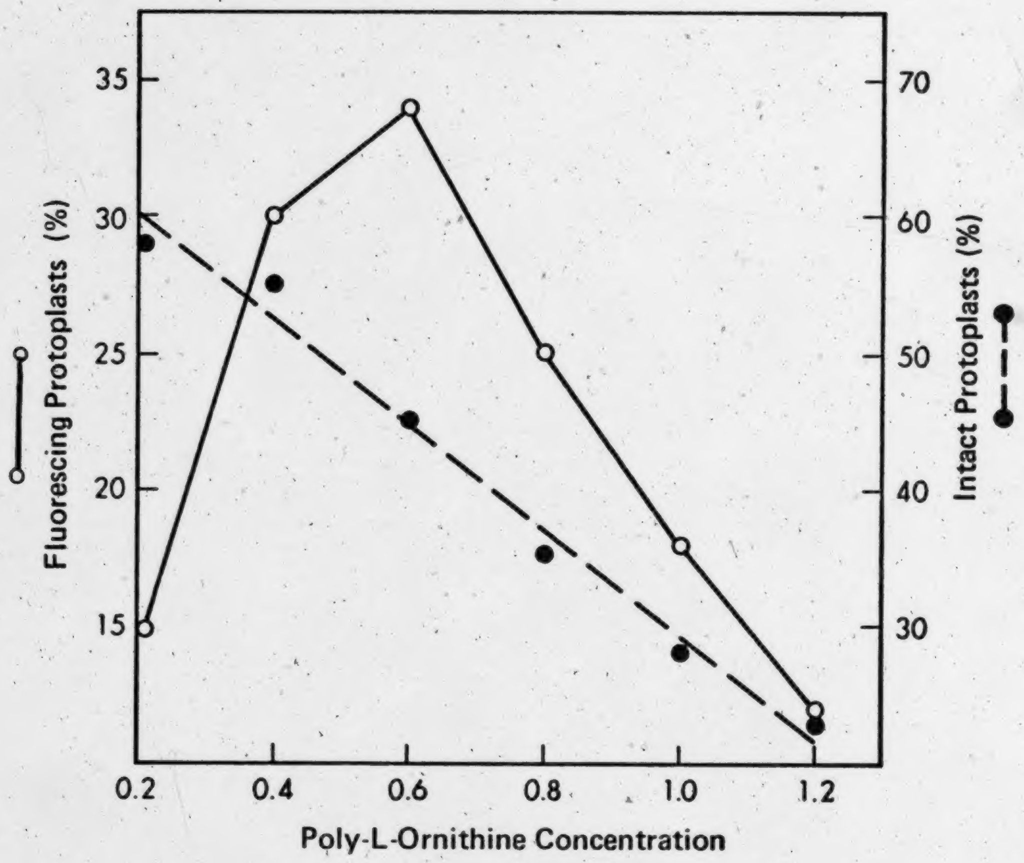

35
30
25
20
15
Fluorescing Protoplasts (%)
70
60
50
40
30
Intact Protoplasts (%)
0.2
0.4
0.6
0.8
1.0
1.2
Poly-L-Ornithine Concentration

Table 8. Effect of pre-incubation of clover yellow mosaic virus with poly-L-ornithine on the frequency of infection in cowpea mesophyll protoplasts

Time (minutes)	Percentage of protoplasts showing fluorescence[a]
0	15
10	24
20	25
30	26
40	28
60	28

[a] Protoplasts were inoculated at a concentration of 5 µg per ml virus and 0.4 µg per ml poly-L-ornithine at pH 5.8. Percentage of protoplasts showing fluorescence was obtained by counting 48 hours after inoculation.

Table 9. Effect of duration of contact of clover yellow mosaic virus with cowpea mesophyll protoplasts on the frequency of infection

Incubation (minutes)	Percentage of protoplasts showing fluorescence[a]
0	9
15	22
30	23
45	23
60	24

[a] Protoplasts were inoculated with 4 μg per ml and 0.4 μg per ml of virus and poly-L-ornithine respectively (pH 6.0). Protoplasts showing fluorescence were counted 48 hours after inoculation.

inoculum concentration of 4 µg per ml, beyond which there was no significant increase in the percentage of infected protoplasts (Figure 12).

5.36 pH of inoculation medium

Potassium citrate buffers with pH's from 4.9 to 6.3 were used to determine the influence of pH on the rate of infection. The highest percentage of infection was obtained at pH 6.0 (Figure 13).

5.37 Time-course study

The time course of CYMV multiplication was studied by assaying the infectivity of protoplasts with increasing incubation periods up to 60 hours (Figure 14). At zero hour, the protoplasts retained a small amount of infectivity, which decreased to about 40% after 6 hours. The infectivity increased rapidly and continuously from 12 hours up to 48 hours of incubation. Thereafter, the virus multiplication ceased. At least a 100-fold increase in virus infectivity was observed during the 60 hours of incubation over the initial infectivity at zero time, as determined by assaying a series of dilutions of the protoplast extracts. Intact virus particles were observed with the electron microscope when protoplast extracts were examined 60 hours after inoculation (Plate 8E).

Infection of protoplasts with CYMV was monitored by using

FIGURE 12 Effect of the concentration of clover yellow mosaic virus on infection of cowpea mesophyll protoplasts. The protoplasts were inoculated at a poly-L-ornithine concentration of 0.4 µg per ml, pH 5.8 and sampled 48 hours after inoculation.

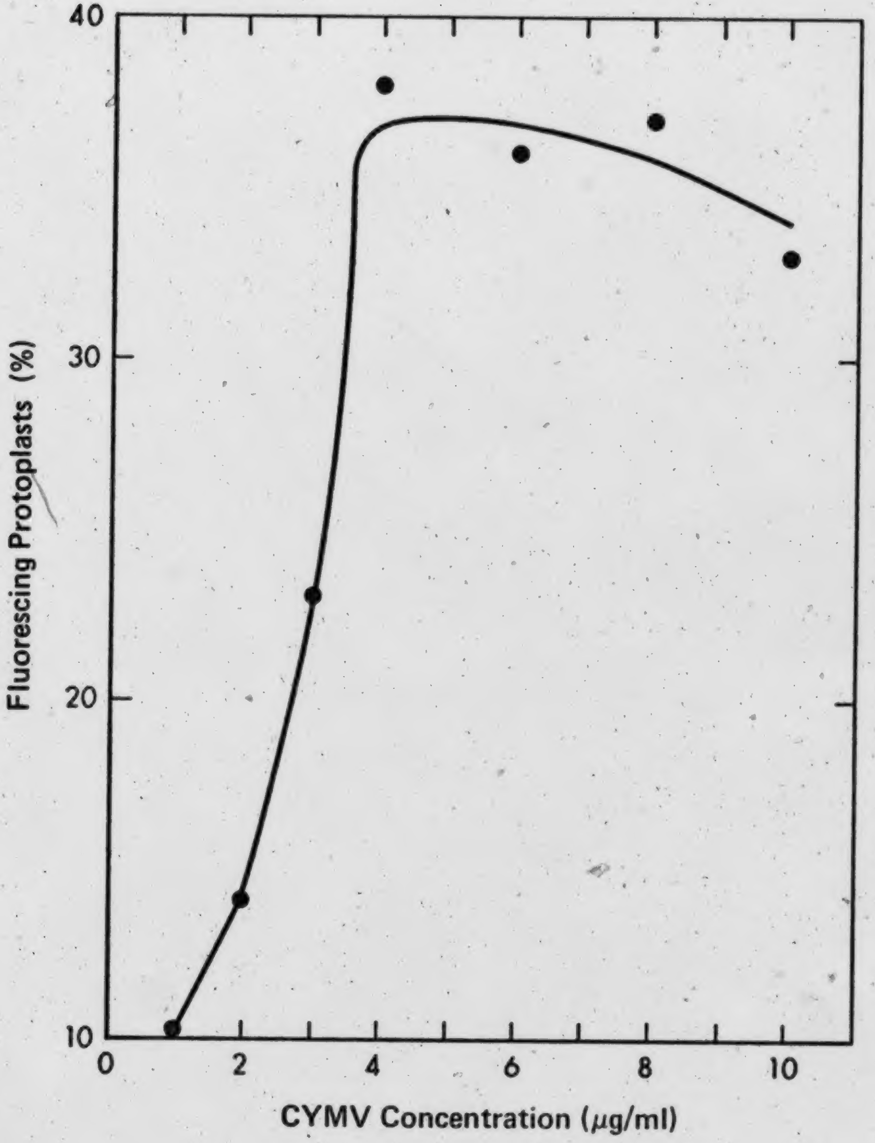

40
30
20
10
Fluorescing Protoplasts (%)
0
2
4
6
8
10
CYMV Concentration (μg/ml)

FIGURE 13 Effect of the pH of inoculum on infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. The protoplasts were inoculated with virus and poly-L-ornithine concentrations of 5 µg per ml and 0.4 µg per ml respectively. Protoplasts were sampled 48 hours after inoculation.

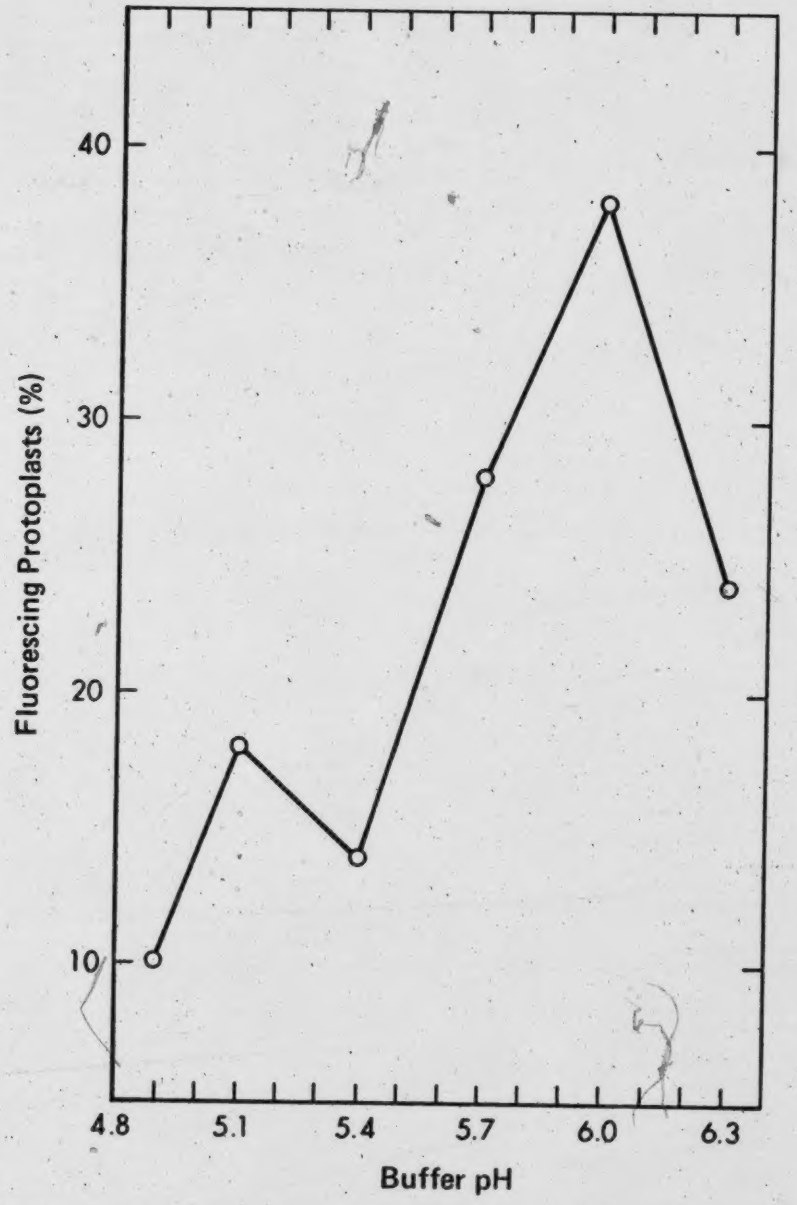

Fluorescing Protoplasts (%)
40
30
20
10
4.8
5.1
5.4
5.7
6.0
6.3
Buffer pH

FIGURE 14 Time-course of the increase of clover yellow mosaic virus in cowpea mesophyll protoplasts, determined by assaying infectivity of extracts of protoplasts at various times after inoculation in *Chenopodium amaranticolor* leaves.

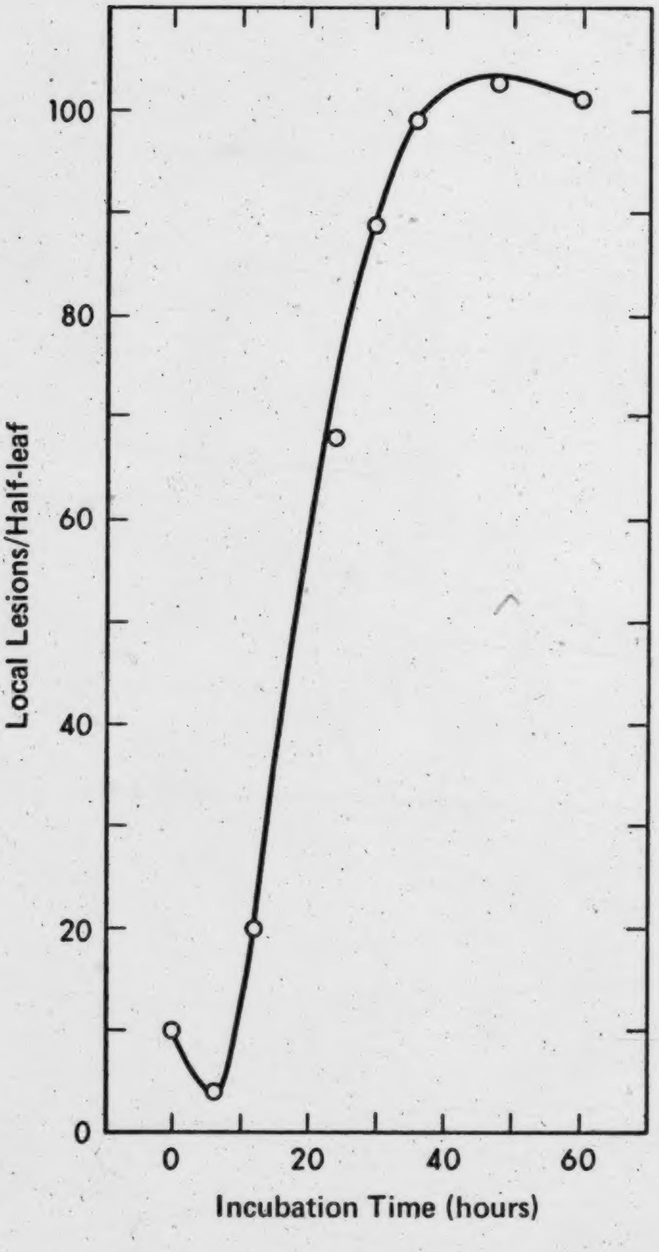

Local Lesions/Half-leaf
0
20
40
60
80
100
0
20
40
60
Incubation Time (hours)

Table 10. Time-course study of cowpea mesophyll protoplasts by the fluorescent antibody staining technique after inoculation with clover yellow mosaic virus

Time after inoculation (hours)	Percentage of protoplasts showing fluorescence[a]
0	0
12	3
24	20
30	28
48	31
60	33

[a] Protoplasts were inoculated with 4 µg per ml virus and 0.4 µg per ml poly-L-ornithine respectively at pH 6.0.

the fluorescent antibody staining technique. The first fluorescence due to CYMV antigen was detected as early as 12 hours of incubation, as small, weak, yellowish green specks in the cytoplasm. The number of infected protoplasts however, was only 3% at this time. Small masses of fluorescence were observed after 24 hours of incubation (Plate 8C), and after 36 hours most of the cytoplasm was occupied by large diffuse masses of fluorescent material. These were apparantly formed by coalescence of the smaller masses observed earlier. Plate 8D shows such a mass occupying most of the protoplast. Chloroplasts were observed as unstained dark bodies. No fluorescence was observed when protoplasts were similarly treated with conjugated normal globulin. Percentages of protoplasts showing fluorescence increased rapidly (from 3% to 31%) from 12 hours to 48 hours incubation, and increased more slowly thereafter (Table 10).

5.40 DISCUSSION

In this investigation, up to 38% of cowpea mesophyll protoplasts were infected by CYMV, under optimal conditions (Figure 13). The time course study, involving the infectivity assay and fluorescent antibody staining, indicated synchronous infection resulting in a single step-growth of CYMV, with a growth curve similar to those reported for other viruses (Takebe and Otsuki, 1969; Hibi and Yora, 1972; Hibi et al., 1975). The amount of virus accumulated in the protoplasts was apparently high, as revealed by the extensive amount of fluorescence observed in the infected protoplasts and by the results of protoplast extract dilutions to 100-fold used in the infectivity assay.

CYMV requires poly-L-ornithine to infect protoplasts. Poly-L-ornithine has been shown to be essential for infecting tobacco protoplasts with several viruses (Aoki and Takebe, 1969; Takebe and Otsuki, 1969; Otsuki and Takebe 1973; Motoyoshi et al., 1973b), Brassica protoplasts with TYMV (Ranaudin et al., 1975) and cowpea protoplasts with TMV (Koike et al., 1976). All these viruses are negatively charged at the pH ranges used for inoculation, whereas positively charged viruses such as PEMV and BMV do not require poly-L-ornithine to infect protoplasts (Motoyoshi and Hull, 1974; Motoyoshi et al., 1974a). However, the negatively charged CPMV and CMV do not require poly-L-ornithine to infect a high percentage of cowpea protoplasts (Hibi et al., 1975; Koike et al., 1976). Thus, the requirement of poly-L-ornithine seems not to be a universal feature of negatively charged viruses.

The evidence, presented for CYMV with regard to pre-incubation of virus with poly-L-ornithine, clearly shows that this virus requires binding with the polycation prior to inoculation (Table 8). These results agree with the previous findings for other viruses (Otsuki et al., 1974; Motoyoshi et al., 1974b). The actual mechanism of poly-L-ornithine is, however, not conclusively known.

The effect may be at the virus level or at the protoplast level, and by a stimulating effect on pinocytosis (Takebe and Otsuki, 1969; Otsuki et al., 1972; Hibi and Yora, 1972) or by causing plasmalemma lesions which presumably serve as sites for virus binding (Burgess et al., 1973a, b).

The results of inoculation with CYMV under the inoculation conditions used in this investigation are strikingly similar to those with PVX, the type-member of the potex virus group for which the optimal conditions for infection have been worked out (Otsuki et al., 1974). The most important similarity is its response to the pH of the inoculation medium. CYMV required an optimal pH of 6.0 for maximum infection, PVX also required a very similar though broader optimal pH (around 5.8). This pH is higher than that for a majority of viruses previously reported. The reason for such a requirement has not been fully explained, although for certain virus-protoplast combinations at least the electrical charge on the virus particle seems to be very important. The isoelectric point of CYMV is reportedly 5.3 (Purcifull and Shepherd, 1964), and in this investigation, infection was low at pH 5.4 (Figure 13). Since the net charge on the virus particles is zero at the isoelectric point, one would expect very little virus adsorption to the surface of the protoplast membrane if this depends on the charge of virus particles. It will therefore be interesting to determine whether, as the pH value increases there are increases in the negative charge of the virus particles, to an optimum at pH 6.0 which for CYMV causes maximum adsorption to the protoplast surface.

CYMV, like PVX, required concentrations of virus inoculum higher than those of certain other viruses for maximum infection (Takebe and Otsuki, 1969; Otsuki and Takebe, 1973). The reason for such a response cannot be attributed to the lower specific infectivity of the virus, since the results indicate that the purified CYMV used as inoculum in this investigation had a reasonably high specific infectivity as judged by the infectivity assay (Figure 10). Therefore, it appears that there is at least an additional factor that is unknown to us at present, which is operable to achieve the maximum infection of cowpea protoplasts with CYMV.

CHAPTER 6

CONCLUDING REMARKS

The natural occurrence of CYMV on field vetch has been reported in this thesis for the first time. Certain serological and symptomatological distinctions have been made between this isolate an~~d oth~~er Canadian isolate tested. Nevertheless, it has not been accorded the status of a strain, to avoid possible confusion, since further detailed biological and physicochemical characterization is desirable before naming it as a strain. Agrawal et al. (1962a) reported that CYMV was more prevalent in the west than in the east after surveys made to determine the distribution of CYMV in the United States. With regard to the distribution of CYMV in Canada, the present study and other studies from eastern and western parts (Pratt, 1961, 1968; Welsh et al., 1973; Gates and Bronskill, 1974) indicate that the virus is also widely distributed only in the western part of Canada. This regionally limited prevalence of the virus, however, is hard to explain on the basis of information available at present. The actual role of vetch in the spread of CYMV is unclear, although it may be safe to state that it can serve as an ideal overwintering host by virtue of its perennial nature, and may be an important source of virus spread to other legumes in Alberta.

The methodology used in the identification and characterization of CYMV, besides providing a better understanding of this virus, has also facilitated the precise handling of the virus required for later studies described in this thesis. Thus the purification by the PEG method has yielded highly purified, infective preparations with consistently high yields, which have proven to be extremely useful in virus multiplication studies both as pure inoculum and as immunogen producing homologous antiserum of high titre. This has permitted, in turn, an exploration of fluorescent antibody technique with CYMV.

Fluorescent antibody technique had only limited usage in plant virology until it was applied in detecting the accumulation of TMV antigen in tobacco protoplasts (Takebe and Otsuki, 1969; Otsuki and Takebe, 1969). The technique is not only useful in a study such as the above, but also convenient for identification of viral inclusions *in vivo*. There are two categories of inclusion bodies, namely 1) inclusions directly related to virus assembly, and 2) those that are not directly related to the virus but occur in host cells in association with virus infection (Shepard and Shalla, 1969; Hiebert et al., 1971). Since these two groups of inclusion bodies cannot be distinguished by ordinary light microscopy, the application of immunofluorescence microscopy, as in this study, will prove extremely useful in future studies related to the identification of inclusions.

In studying virus infection of protoplasts, a combined use of the infectivity test, fluorescent antibody technique and electron microscopy is common, and a dependable infectivity assay technique is particularly important in determining rates of virus infection and multiplication. This is clearly seen in time-course studies of certain virus-protoplast systems (Otsuki and Takebe, 1973; Shalla and Petersen, 1973), where the reliability of the data suffers from the use of an assay technique of low sensitivity. In the present CYMV study, a highly efficient infectivity assay system has been developed by testing and eliminating a number of the factors reducing its accuracy. This system is now capable of detecting even the low infectivity present at zero and 6 hours after inoculation of protoplasts by CYMV.

In cowpea mesophyll protoplasts, synchronous infection of CYMV has been successfully demonstrated. While this achievement has been used here only to provide information concerning the optimal conditions for infection, it constitutes a significant advance over current procedures and holds a considerable promise for future study. Detailed studies concerning the process of CYMV infection and multiplication, including the sequential synthesis of viral RNA and protein, can be made, as has recently been demonstrated with TMV (Paterson and Knight, 1975). In this regard, the process of virus inclusion formation may be studied in detail, and its exact role in virus multiplication, about which very little is now known, may be determined. A second possibility is the study of viral interaction within the host cell, where synchronous infection is essential. It would be extremely interesting to understand the mode of interaction, if any, between CYMV and WCMV, as they are known to occur often as a complex in naturally infected legume hosts. A third and significant possibility is the study of antiviral compounds. Here a reliable synchronous infection of protoplast system would greatly facilitate experimental tests to determine the basic mechanisms of virus inhibition or inactivation due to these compounds at cellular and molecular levels. Finally, the protoplast system may be useful in the development of virus-resistant breeding lines. This may be accomplished by regenerating plants from protoplasts, a routine procedure with several species of plants (Gamborg and Wetter, 1975). The protoplasts are inoculated initially, with virus and cultured. These cultured protoplasts will regenerate cell walls, and the resultant cell cultures may be maintained indefinitely. The cells that are inherently resistant to virus, or the mutant cells that have acquired resistance to virus infection, may be selected by repeatedly challenging with virus and then regenerating desired cell lines. Eventually it may be possible to secure plants resistant or immune to virus infection.

PLATES AND LEGENDS

PLATE 1. Symptoms of plants infected by the vetch isolate of clover yellow mosaic virus.

A. Systemic symptoms on field vetch (Vicia sativa L.). Left, healthy leaf; middle, an older leaf showing mosaic with some necrosis; right, younger leaf showing drastic reduction in leaf size, seven weeks after inoculation.

B. A comparison of symptoms of field vetch (Vicia sativa L.) infected by the three isolates of clover yellow mosaic virus. Left, CYMV-H isolate showing mild mosaic; middle, the vetch isolate showing necrosis associated with severe mosaic; right, CYMV-S isolate showing necrosis and defoliation.

C. Alaska pea (Pisum sativum L.) showing mosaic symptoms, three weeks after inoculation.

D. Systemic symptoms on a trifoliate leaf of Red Kidney bean (Phaseolus vulgaris L.), six weeks after inoculation.

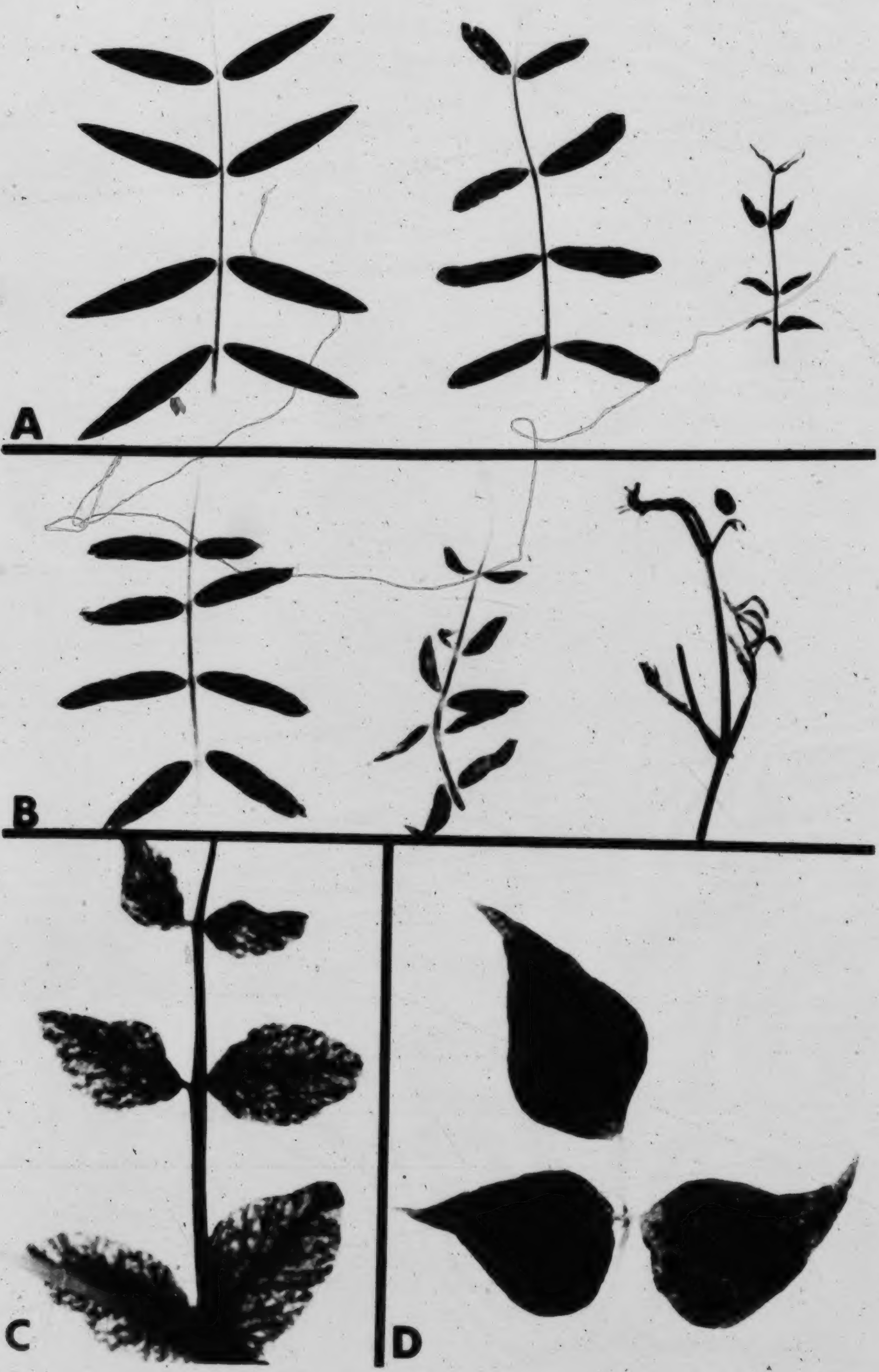

A
B
C
D

PLATE 2. Symptoms of plants infected by the vetch isolate of clover yellow mosaic virus.

A and B. Systemic and local symptoms on broad bean (*Vicia faba* L. 'Broad Windsor')

A. Mosaic symptoms in systemically infected leaves, four weeks after inoculation.

B. Solid local necrotic lesions produced one week after inoculation.

C. Systemic mosaic symptoms on alsike clover (*Trifolium hybridum* L.). Severe symptoms on an old leaf (middle) and mild symptoms on a young leaf (right). The leaf on the left is an uninoculated control.

D and E. Healthy (D) and infected leaves (E) of white clover (*Trifolium repens* L.). A trifoliate leaf showing systemic mosaic symptoms, six weeks after inoculation.

F. A half-leaf of *Chenopodium amaranticolor* Coste & Reyn., showing local lesions, nine days after inoculation.

G. Local lesions on *Gomphrena globosa* L., two weeks after inoculation. The leaf on the left is an uninoculated control.

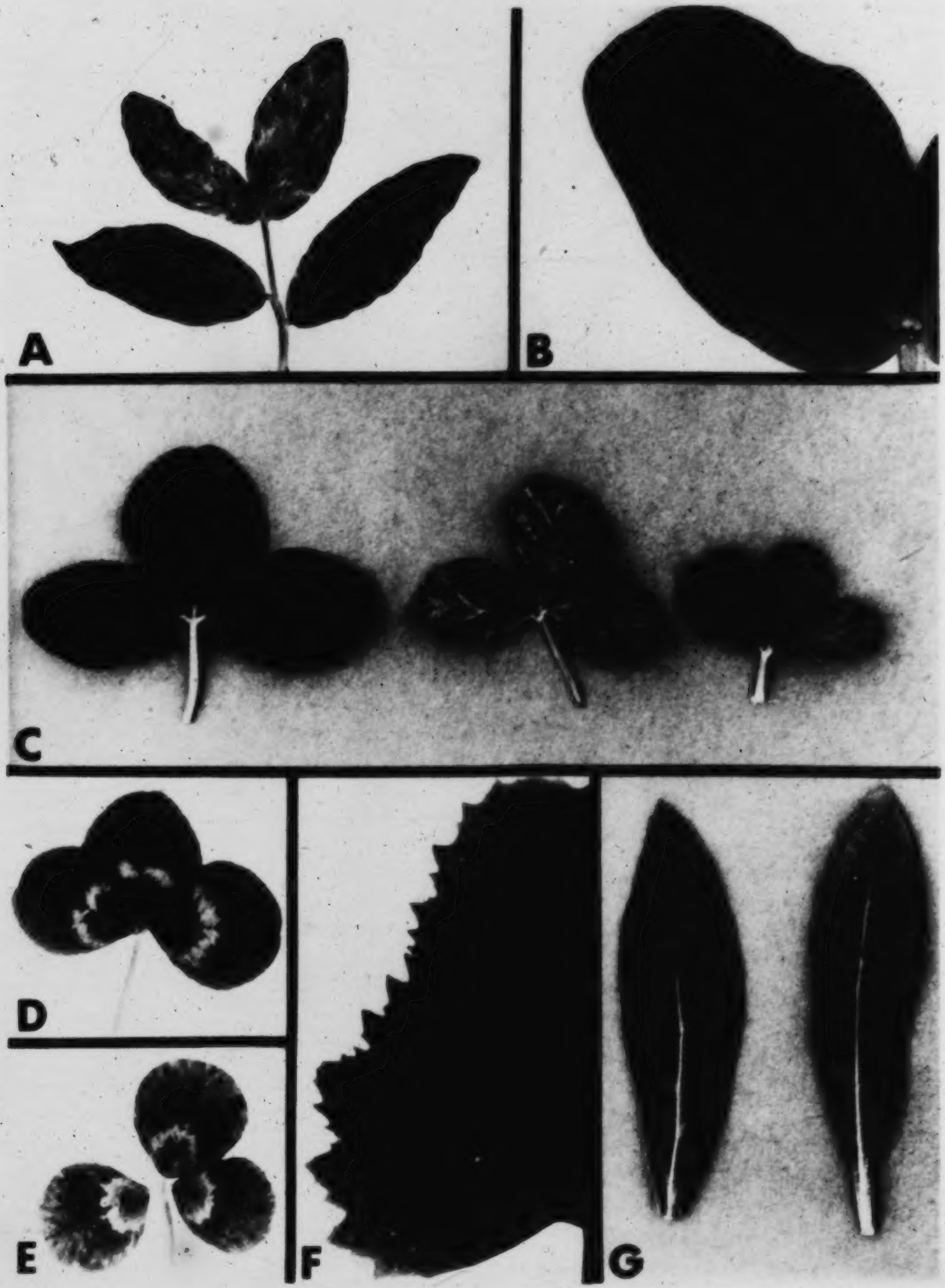

A
B
C
D
E
F
G

PLATE 3. Photomocrographs of inclusion bodies produced in the epidermal cells after inoculation with the vetch isolate of clover yellow mosaic virus.

A. Two amorphous inclusions, seen as corner deposits (indicated by arrows) in the infected epidermal cells of broad bean (*Vicia faba* L. 'Broad Windsor'), one week after inoculation. Bar represents 5 μm.

B. A large amorphous inclusion body (indicated by arrows) in the infected epidermal cells of pea (*Pisum sativum* L. 'Alaska'), one week after inoculation. N, nucleus. Bar represents 5 μm.

C. Parallel bands (indicated by arrows) in the infected epidermal cells of pea (*Pisum sativum* L. 'Alaska'), two weeks after inoculation. Bar represents 5 μm.

D. A crystalline inclusion, hexagonal in shape, in an infected epidermal cell of broad bean (*Vicia faba* L. 'Broad Windsor'), two weeks after inoculation. Bar represents 2 μm.

E and F. Fluorescence micrographs of the infected epidermal cells of cowpea (*Vigna sinensis* Savi 'Black Eye'). Under UV light, the amorphous inclusions (indicated by arrows) in the corners fluoresce as yellowish green masses after having been stained with anti-CYMV globulin labelled with fluorescein isothiocyanate. Bar represents 10 μm.

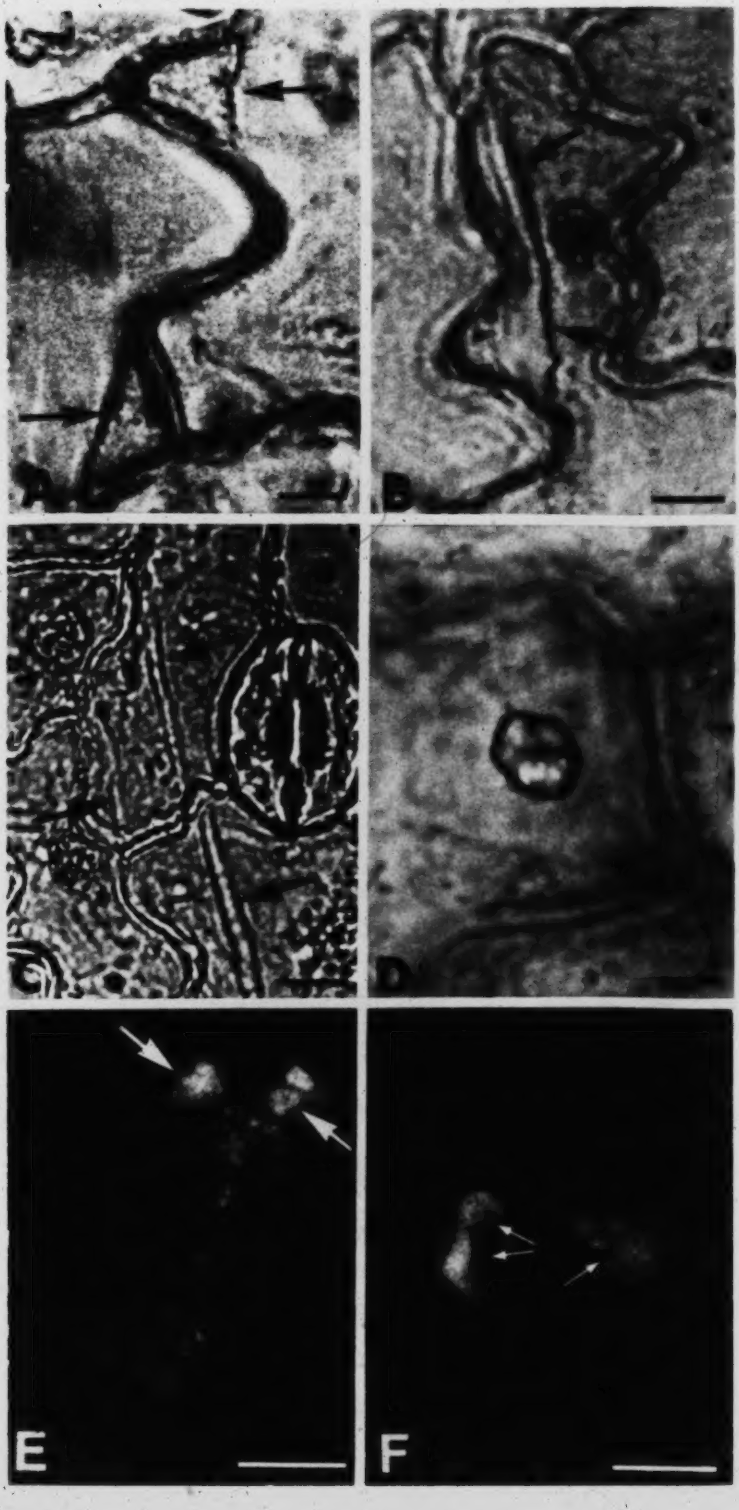

A
B
C
D
E
F

PLATE 4. Low magnification electron micrographs of thin sections of leaf tissues from field vetch (Vicia sativa L.), two weeks after inoculation with the vetch isolate of clover yellow mosaic virus (Abbreviations used: CH, chloroplast; N, nucleus; V, virus aggregate; VA, vacuole. Bars represent 3 µm).

A. A longitudinal section of mesophyll cells. Note a virus aggregate across an infected cell (indicated by an arrow).

B. Transverse section of mesophyll cells. Virus aggregate is seen across an infected cell (indicated by arrows).

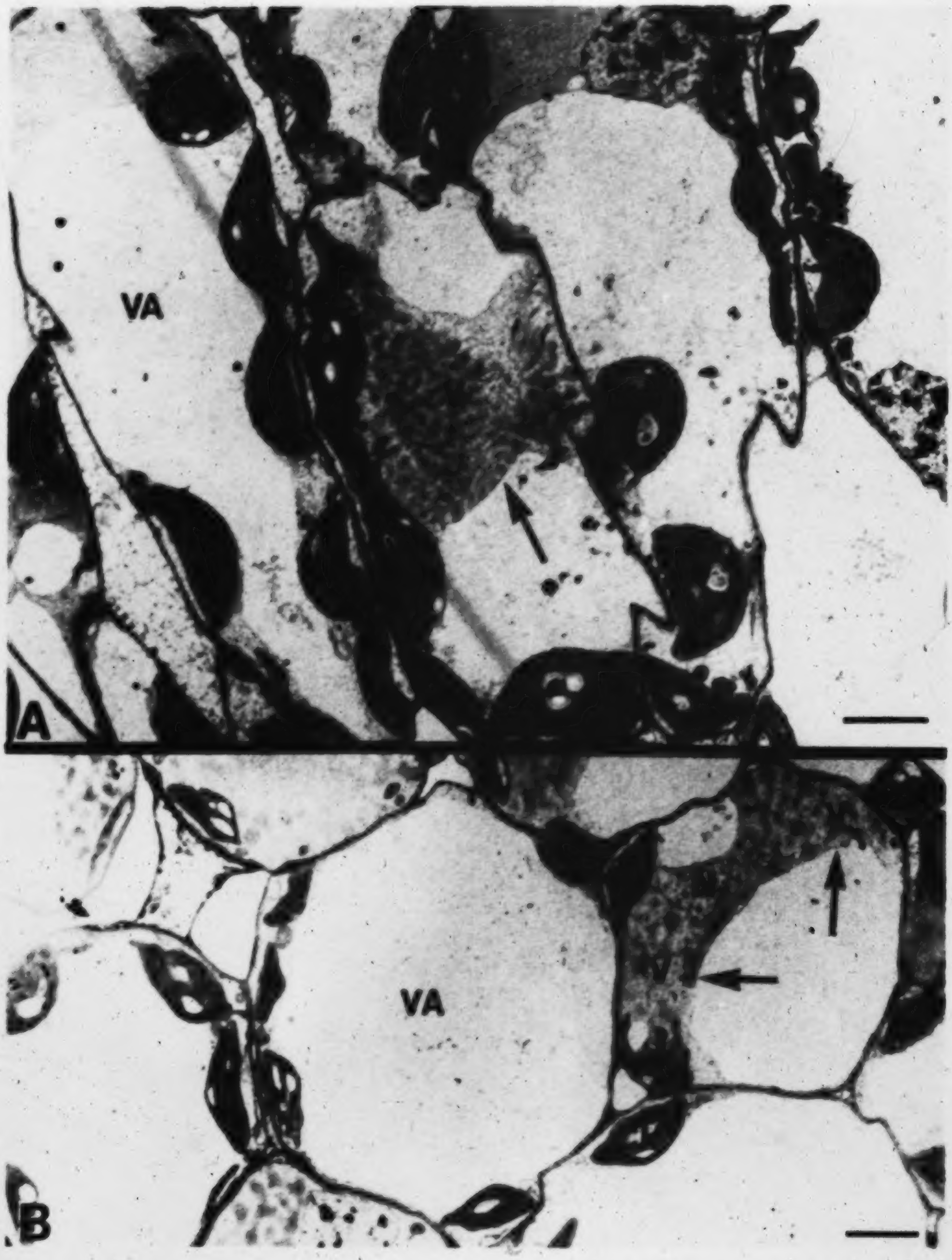

VA
A
VA
B

PLATE 5. Virus aggregates in thin sections of leaf tissues of field vetch (Vicia sativa L.), two weeks after inoculation with the vetch isolate of clover yellow mosaic virus (Abbreviations used: CH, chloroplast; CW, cell wall; V, virus aggregate. Bars represent 1 µm).

A and B. Transverse sections of mesophyll cells containing large aggregates at the periphery of cells.

C. A transverse section of mesophyll cell showing a long strip of dense and tightly packed aggregate of virus particles.

A
B
C
CH

PLATE 6. A transverse section of mesophyll cells of field vetch (Vicia sativa L.), showing large virus aggregates. Note spiral aggregates in the top cell. CH, chloroplast; S, starch grain; V, virus aggregate; VA, vacuole. Bar represents 1 μm.

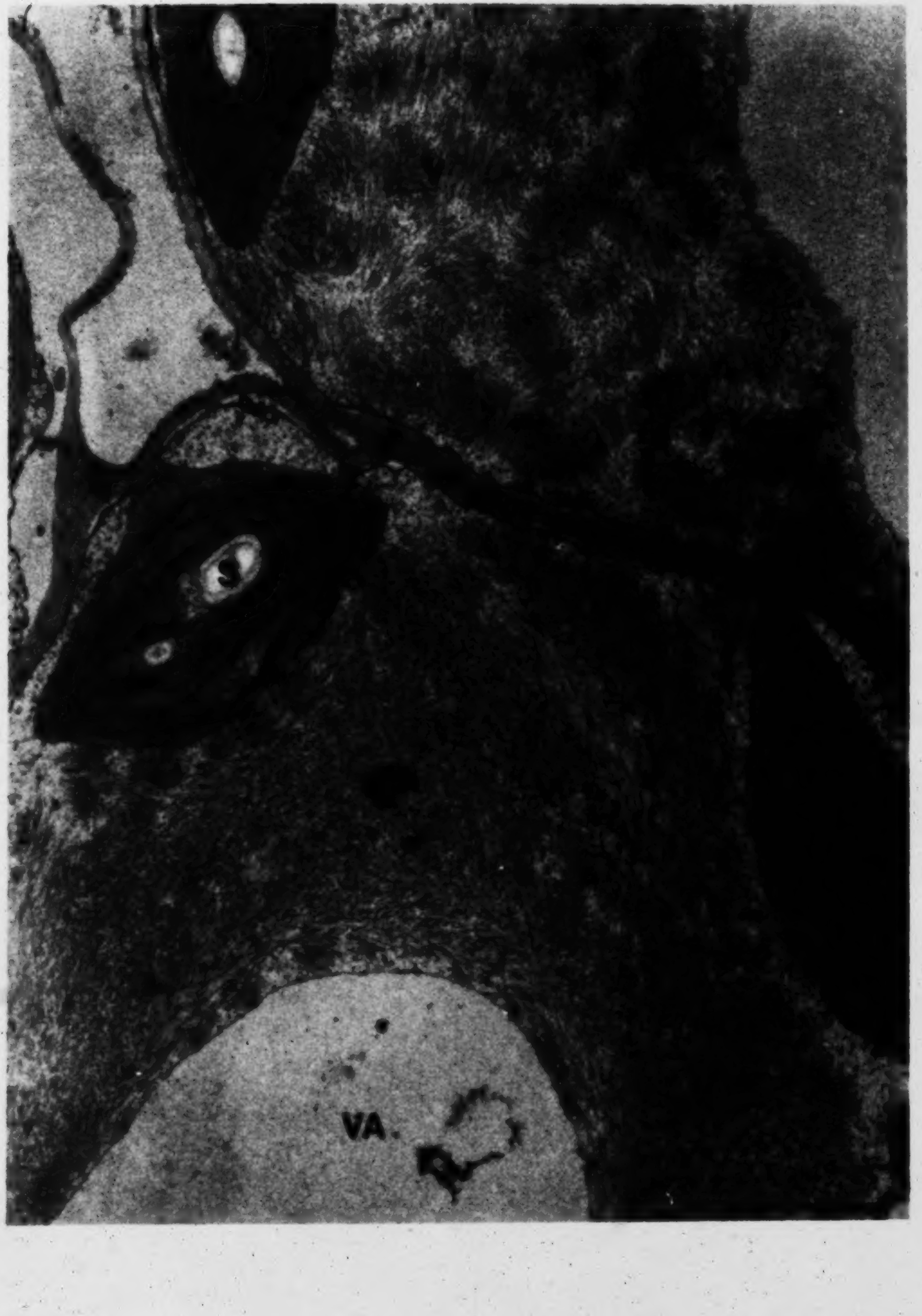

S
VA

PLATE 7.

A and B. Electron micrographs of the vetch isolate of clover yellow mosaic virus. Virus particles were stained with 2% aqueous phosphotungstic acid, pH 7.0.

A. Negatively stained leaf-dip preparation obtained from infected pea leaves (Pisum sativum L. 'Alaska'). Bar represents 1 µm.

B. Negatively stained preparation of highly purified virus obtained by the polyethylene glycol (PEG) method. Bar represents 0.5 µm.

C. A centrifuge tube with cesium chloride gradient, showing a light scattering band representing clover yellow mosaic virus (the vetch isolate), after centrifugation at 100,000 g for 20 hours.

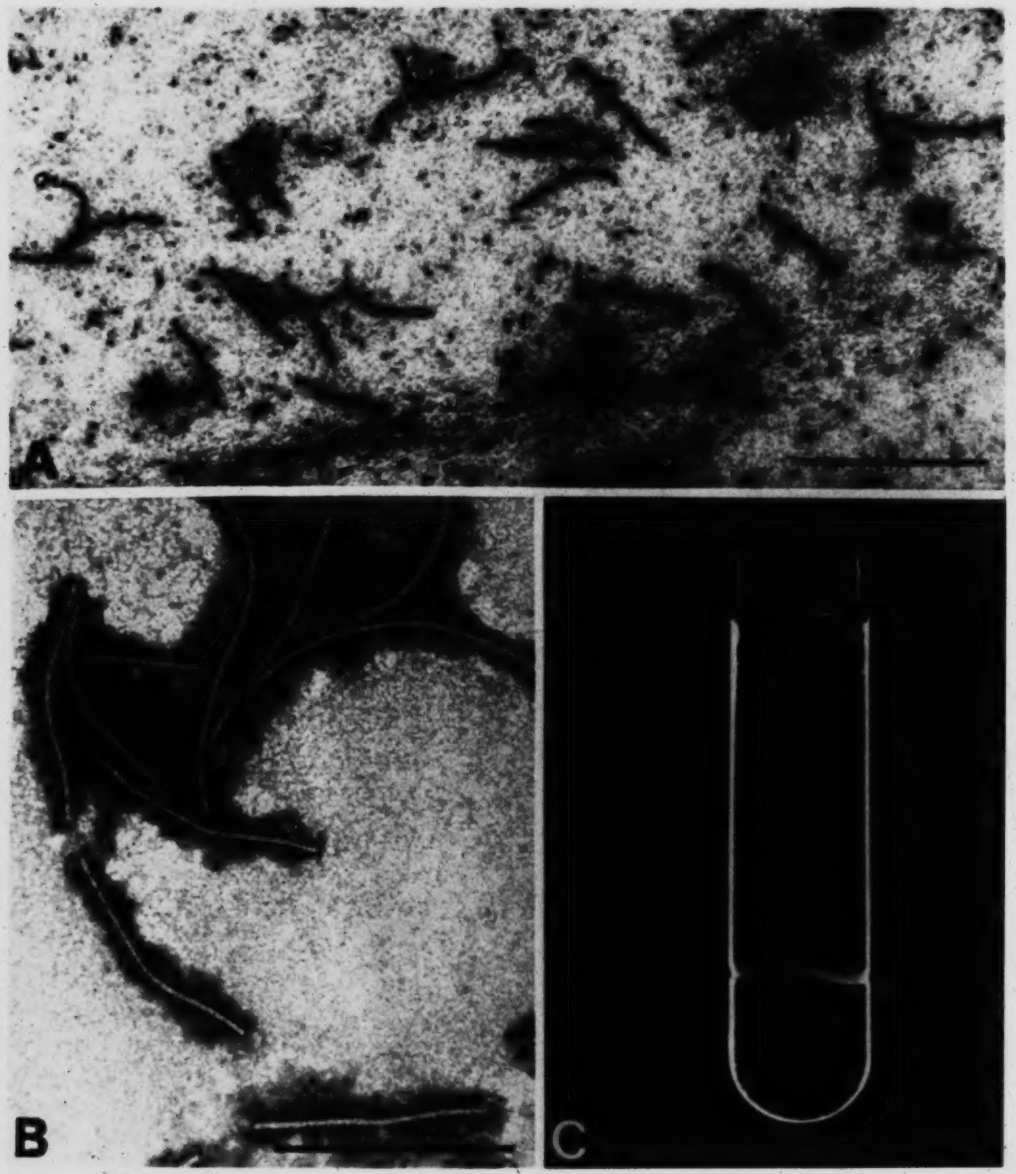

A
B
C

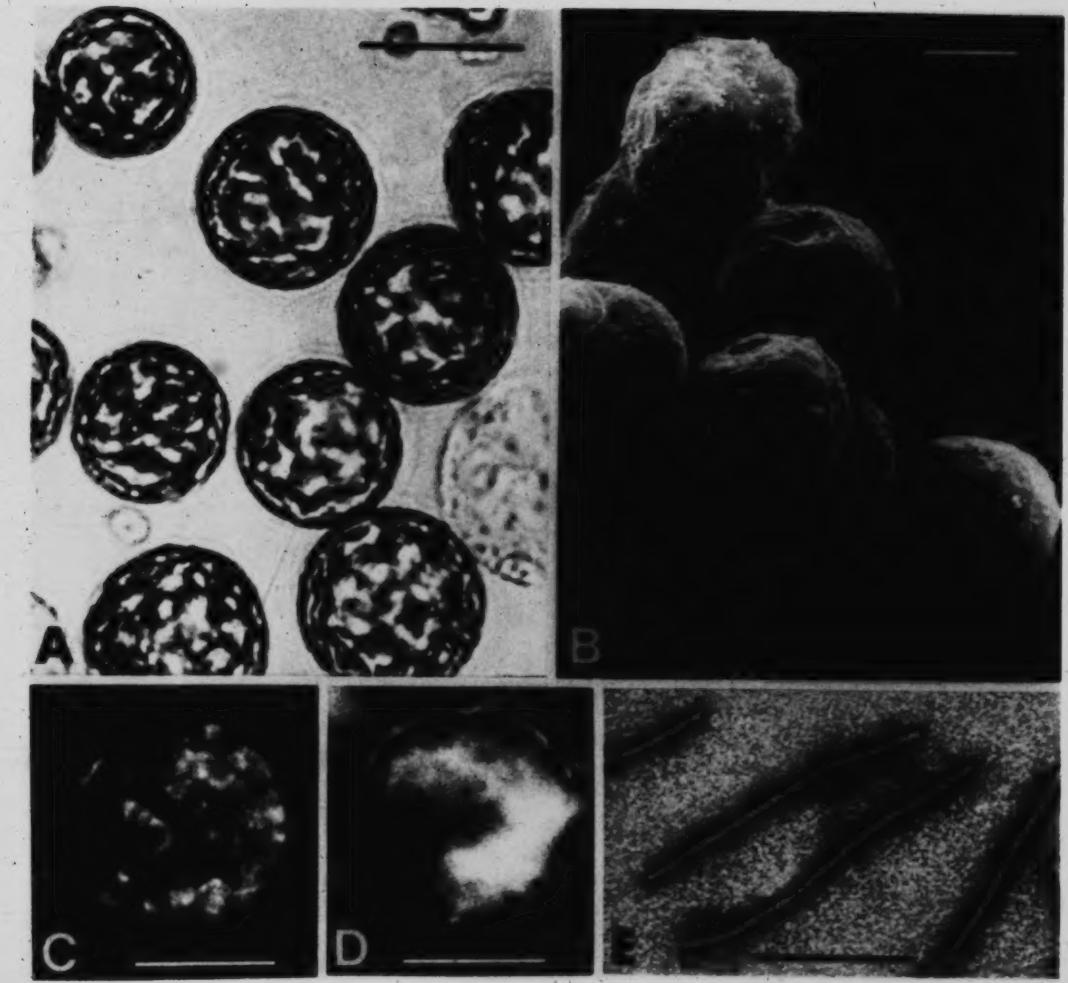
PLATE 8.

A. A photomicrograph of cowpea (Vigna sinensis Savi 'Black Eye') mesophyll protoplasts sampled 12 hours after incubation. Bar represents 20 µm.

B. A scanning electron micrograph of cowpea mesophyll protoplasts sampled 12 hours after incubation. Bar represents 10 µm.

C and D. Fluorescence micrographs of cowpea mesophyll protoplasts sampled 24 hours and 36 hours respectively after inoculation with clover yellow mosaic virus. Fluorescence was observed as small masses 24 hours after inoculation later coalesced to form a large mass 36 hours after inoculation. Chloroplasts remained unstained and were observed as dark bodies. Bar represents 15 µm.

E. Virus particles obtained from a 100-fold dilution of an extract of cowpea mesophyll protoplasts inoculated with clover yellow mosaic virus. The extract was obtained from protoplasts, sampled 60 hours after inoculation, and negatively stained with 2% aqueous phosphotungstic acid, pH 7.0. Bar represents 0.2 µm.


A
B
C
D
E

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APPENDIX

Table 11. Lesion counts: Time-course of increase in local lesion number in Chenopodium amaranticolor leaves inoculated with clover yellow mosaic virus (cf. Figure 6 in the text)

	Time after inoculation (days)							
	4	5	6	7	8	9	10	12
Lesion counts/ leaf	22	46	57	72	83	86	86	88
	13	28	41	62	72	74	75	76
	9	26	30	38	66	66	66	68
	8	29	31	52	58	59	59	59
	10	24	52	68	75	75	76	76
	12	34	49	69	76	80	88	88
	7	18	32	48	51	53	53	58
	9	26	56	66	71	75	78	80
	10	30	35	52	68	71	72	72
	14	49	51	82	91	91	91	92
	11	22	34	51	65	69	69	69
	6	36	39	60	69	76	77	77
	21	43	60	84	90	94	95	95
	28	41	57	79	79	80	83	83
	4	12	22	49	54	62	62	63
	16	20	33	59	73	77	79	79
Total	198	484	680	991	1141	1188	1209	1223
Mean	12.37	30.25	42.43	61.93	71.31	74.25	75.56	76.43

Table 12. Lesion counts: Number of local lesions incited by clover yellow mosaic virus in the leaves of Chenopodium amaranticolor at different leaf positions (cf. Table 5 in the text)

	Leaf position							
	7	8	9	10	11	12	13	14
	45	53	60	65	70	82	65	47
	58	67	78	84	78	97	70	59
	68	70	85	78	82	99	81	60
Lesion counts/ leaf	46	79	62	82	74	90	80	48
	78	51	69	56	76	82	63	43
	42	82	73	85	77	74	50	31
	31	73	68	86	81	71	56	25
	25	50	52	56	71	70	53	32
	36	85	82	91	89	95	54	56
	49	72	71	64	76	80	60	54
Total	478	682	700	748	771	840	632	455
Mean	47.80	68.20	70.00	74.80	77.10	84.00	63.20	45.50

Table 13. Lesion counts: A comparison of local lesion number obtained in *Chenopodium amaranticolor* leaves incubated at 17°C, 21°C, 25°C and 29°C after inoculation with clover yellow mosaic virus (Effect of post-inoculation temperature Exp. I, cf. Figure 7 and Table 6 in the text)

	Time after inoculation (days)							
	6	7	8	9	10	11	12	14
	2	16	26	48	55	62	66	70
	6	23	28	46	62	67	75	78
17°C	9	14	26	40	57	72	79	79
	8	26	39	51	66	69	82	86
Lesion	3	9	18	30	59	63	65	75
counts/	5	13	22	36	50	59	60	63
leaf	6	17	25	42	52	68	80	89
	11	14	19	33	49	54	59	64
	7	16	21	48	60	70	73	81
	2	6	16	28	35	42	48	56
	9	19	29	55	65	73	74	80
	14	21	30	49	61	74	76	85
Total	82	194	299	506	671	773	837	906
Mean	6.83	16.16	24.91	42.16	55.91	64.41	69.75	75.50

	4	5	6	7	8	9	10	12
	10	38	53	80	86	91	93	93
	16	42	58	81	92	96	98	98
21°C	22	41	62	88	97	102	106	109
	5	18	40	76	88	89	94	99
Lesion	14	22	38	68	74	82	84	84
counts/	16	28	39	52	66	75	80	82
leaf	6	14	44	58	61	69	70	71
	8	12	28	47	59	62	66	69
	13	26	52	78	90	95	95	95
	12	24	49	71	79	86	87	87
	9	18	32	60	79	81	81	81
	11	28	61	90	93	99	101	103
Total	142	311	556	849	955	1027	1055	1071
Mean	11.83	25.91	46.33	70.75	79.58	85.58	87.91	89.25

Table 13. Continued

	Time after inoculation (days)				
	4	5	6	7	8
	26	59	76	99	102
	38	71	92	117	122
	16	38	52	86	89
25°C	21	52	61	72	72
	36	48	75	85	92
Lesion	18	31	43	67	68
counts/	46	64	94	108	111
leaf	12	26	52	64	66
	8	29	59	94	97
	13	42	60	74	74
	28	49	80	95	99
	22	34	72	106	109
Total	284	543	816	1065	1101
Mean	23.61	45.20	68.00	88.92	91.00
		3	4	5	
		23	58	77	
		46	82	122	
		12	50	110	
29°C		41	75	80	
		16	46	82	
Lesion		39	79	106	
counts/		44	69	140	
leaf		18	38	101	
		25	36	86	
		29	79	81	
		46	66	125	
		45	46	59	
Total		384	724	1169	
Mean		32.00	60.30	97.42	

Table 14. Lesion counts: Effect of post-inoculation temperature on the number of local lesions incited by clover yellow mosaic virus in *Chenopodium amaranticolor* leaves Exp. II (cf. Table 6 in the text)

	Temperature (°C)			
	17	21	25	29
Lesion counts/ leaf	20	51	36	31
	25	70	39	36
	29	60	54	81
	40	100	59	53
	48	56	80	85
	60	91	91	50
	41	37	98	99
	24	44	61	76
	39	60	64	102
	43	71	50	90
	56	43	49	101
	31	47	60	74
	36	95	68	108
	33	46	82	89
	22	58	62	96
	28	42	48	75
Total	575	971	1001	1246
Mean	35.94	60.69	62.56	77.88
Standard error	2.95	4.94	4.42	5.95

Table 15. Lesion counts: Effect of post-inoculation temperature on the number of local lesions incited by clover yellow mosaic virus in *Chenopodium amaranticolor* leaves Exp. III (cf. Table 6 in the text)

	Temperature (°C)			
	17	21	25	29
Lesion counts/ leaf	69	129	128	140
	99	121	108	122
	60	119	110	116
	82	89	91	128
	53	86	82	91
	88	60	86	126
	103	109	125	135
	106	122	120	90
	81	113	106	141
	42	96	96	131
	61	93	103	96
	80	80	81	118
Total	944	1217	1332	1434
Mean	77.00	101.42	103.00	119.50
Standard error	5.89	5.99	4.68	5.24

Table 16. Lesion counts: Effect of pre-inoculation dark treatment on the number of local lesions incited by clover yellow mosaic virus in *Chenopodium amaranticolor* leaves Exp. I

	Time of dark treatment (hours)			
	0 (control)	24	48	72
Lesion counts/ leaf	79	85	50	66
	82	84	52	104
	67	77	67	93
	93	56	83	110
	50	74	56	45
	81	81	59	40
	46	83	50	63
	59	71	71	56
	87	68	92	85
	86	90	85	64
	94	99	83	60
	61	93	84	51
Total	885	961	832	837
Mean	73.75	80.08	69.33	69.75
Standard error	4.78	3.40	4.51	6.62

Table 17. Lesion counts: Effect of pre-inoculation dark treatment on the number of local lesions incited by clover yellow mosaic virus in *Chenopodium amaranticolor* leaves Exp. II

	Time of dark treatment (hours)			
	0 (control)	24	48	72
Lesion counts/ leaf	61	60	45	82
	80	91	56	93
	68	85	59	51
	88	50	91	56
	21	90	63	85
	25	87	81	91
	85	65	40	80
	65	80	83	41
	89	30	87	41
	95	43	90	56
	60	50	45	35
	67	69	43	50
Total	804	800	783	761
Mean	67.00	66.67	65.25	63.42
Standard error	6.84	5.89	5.77	6.14

Table 18. Effect of dark treatment: Results of Duncan's Multiple Range Tests

	Time of dark treatment (hours)	Mean	Designation*
Exp. I	24	80.1	a
	0 (control)	73.8	a
	72	69.8	a
	48	69.3	a
Exp. II	0	67.0	a
	24	66.7	a
	48	65.3	a
	72	63.4	a

* Treatment means not having a common letter in their designation are significantly different at the 5% level of significance.

Table 19. Lesion counts: Effect of light intensity on the number of local lesions in Chenopodium amaranticolor leaves inoculated with clover yellow mosaic virus Exp. I

	Light intensity (lux)			
	6,456	10,760	16,140	21,520
Lesion counts/ leaf	57	68	42	20
	62	53	29	16
	31	82	56	12
	45	91	41	8
	52	68	33	24
	38	55	28	31
	69	45	26	15
	72	59	25	8
	38	66	23	16
	46	75	35	26
	59	85	43	25
	61	89	46	23
	60	49	51	21
	58	56	32	14
	55	51	39	10
	49	58	33	11
Total	852	1050	582	280
Mean	53.25	65.63	36.38	17.50
Standard error	2.85	3.72	2.40	1.75

Table 20 Lesion counts: Effect of light intensity on the number of local lesions in *Chenopodium amaranticolor* leaves inoculated with clover yellow mosaic virus Exp. II

	Light intensity (lux)			
	6,456	10,760	16,140	21,520
Lesion counts/ leaf	52	52	26	22
	30	58	28	12
	51	41	32	8
	72	86	41	9
	35	31	69	17
	39	49	52	7
	32	53	33	7
	40	56	20	8
	21	70	26	20
	66	29	48	9
	51	38	40	6
	42	60	29	15
Total	531	623	444	140
Mean	44.25	51.92	37.00	11.67
Standard error	4.29	4.68	4.00	1.59

Table 21. Lesion counts: Effect of light intensity on the number of local lesions in *Chenopodium amaranticolor* leaves inoculated with clover yellow mosaic virus Exp. III

	Light intensity (lux)			
	6,456	10,760	16,140	21,520
Lesion counts/ leaf	58	26	25	15
	16	58	30	6
	21	51	37	8
	78	56	35	18
	27	29	41	14
	11	40	42	12
	39	25	38	16
	18	58	36	13
	25	39	31	5
	59	71	28	29
	75	22	25	21
	22	36	24	22
	56	34	34	15
	12	59	33	19
	19	28	31	16
	30	50	30	22
Total	566	682	520	251
Mean	35.34	42.63	32.50	15.69
Standard error	5.62	3.76	1.38	1.57

Table 22. Effect of light intensity: Results of Duncan's Multiple Range Tests (cf. Table 7 in the text)

	Light intensity (lux)	Mean	Designation*
Exp I	10,760	65.6	a
	6,456	53.3	b
	16,140	36.4	c
	21,520	17.5	d
Exp II	10,760	51.9	a
	6,456	44.3	ab
	16,140	37.0	b
	21,520	11.7	c
Exp III	10,760	42.6	a
	6,456	35.3	a
	16,140	32.5	a
	21,520	15.7	b

* Treatment means not having a common letter in their designation are significantly different at the 5% level of significance.

Table 23. Lesion counts: Effect of different molar concentrations of phosphate buffer, pH 7.0, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions Exp. I

	Buffer molarity					
	0.5	0.1	0.05	0.01	0.005	Distilled water
Lesion counts/ half-leaf	8	16	32	21	52	15
	35	27	38	68	48	36
	10	31	39	36	47	55
	25	22	36	42	46	8
	13	36	41	58	39	39
	24	15	30	19	42	11
	9	41	36	54	46	40
	22	11	34	30	32	21
	6	28	37	48	56	22
	9	15	40	29	41	38
	33	31	56	51	38	16
	7	8	41	50	49	32
Total	201	281	460	506	536	333
Mean	16.75	23.42	38.33	42.17	44.67	27.75
Standard error	3.03	3.02	1.89	4.42	1.90	4.14

Table 24. Lesion counts: Effect of different molar concentrations of phosphate buffer, pH 7.0, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions Exp. II

	Buffer molarity					
	0.5	0.1	0.05	0.01	.005	Distilled water
Lesion counts/ half-leaf	10	48	71	55	81	20
	18	46	32	58	39	48
	15	51	66	51	69	19
	23	40	51	59	59	49
	26	45	25	49	32	48
	35	44	29	61	77	15
	52	41	62	48	41	13
	34	46	28	56	64	51
	39	38	45	59	78	25
	58	39	27	52	36	52
	46	34	49	47	29	17
	28	38	67	53	71	32
Total	384	510	552	648	676	389
Mean	32.00	42.50	46.00	54.00	56.33	32.42
Standard error	4.30	1.43	5.05	1.36	5.66	4.60

Table 25. Lesion counts: Effect of different molar concentrations of phosphate buffer, pH 7.0, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions Exp. III

	Buffer molarity					
	0.5	0.1	.05	.01	.005	Distilled water
	56	32	73	78	83	26
	20	40	51	91	92	29
Lesion counts/ half-leaf	18	51	42	86	101	48
	12	15	56	48	96	56
	26	14	45	59	42	23
	28	46	52	66	93	36
	34	29	70	75	70	41
	46	19	33	81	58	39
	52	42	40	43	57	37
	13	36	58	60	76	30
	23	20	50	64	75	34
	22	60	49	74	77	52
Total	350	404	619	825	920	451
Mean	29.17	33.67	51.58	68.75	76.67	37.58
Standard error	4.28	4.28	3.35	4.26	5.14	2.97

Table 26. Effect of buffer molarity: Results of Duncan's Multiple Range Tests (cf. Figure 8 in the text)

	Molarity	Mean	Designation*
Exp. I	.005	44.7	a
	.01	42.2	a
	.05	38.3	a
	Distilled water	27.8	b
	0.1	23.4	bc
	0.5	16.8	c
Exp. II	.005	56.3	a
	.01	54.0	ab
	.05	46.0	ab
	0.1	42.5	bc
	Distilled water	32.4	c
	0.5	32.0	c
Exp. III	.005	76.7	a
	.01	68.8	a
	.05	51.6	b
	Distilled water	37.6	c
	0.1	33.7	c
	0.5	29.2	c

* Treatment means not having a common letter in their designation are significantly different at the 5% level of significance.

Table 27. Lesion counts: Effect of pH of 0.01 M phosphate buffer, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions Exp. I

	Buffer pH							
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Lesion counts/ half-leaf	14	32	58	32	42	21	25	19
	16	45	15	72	41	55	26	8
	19	11	22	41	55	38	56	16
	25	22	11	46	52	23	21	20
	26	46	90	25	60	76	18	14
	38	28	9	73	38	38	10	15
	22	12	46	26	31	39	24	17
	15	38	29	65	72	46	23	21
	30	25	86	18	31	82	59	26
	21	32	22	19	38	21	16	22
	7	15	33	80	20	36	15	14
	39	21	21	38	70	69	31	11
	6	16	65	79	32	27	13	12
	42	57	19	28	39	23	66	16
	17	26	49	26	51	25	20	13
	28	19	31	40	56	61	58	15
Total	365	445	606	708	728	680	481	259
Mean	22.81	27.81	37.88	44.25	45.50	42.50	30.06	16.19
Standard error	2.68	3.31	6.37	5.54	3.67	5.09	4.64	1.14

Table 28. Lesion counts: Effect of pH of 0.01 M phosphate buffer, used to extract clover yellow mosaic virus from the infected pea tissue, upon the number of local lesions Exp II

	Buffer pH							
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Lesion counts/ half-leaf	10	16	66	59	49	29	26	9
	20	58	38	60	42	76	25	10
	9	12	31	38	61	52	31	10
	3	31	19	48	56	58	18	12
	16	19	16	41	42	22	28	6
	14	18	52	56	31	16	29	18
	40	55	25	31	38	28	33	16
	46	18	68	52	40	72	21	14
	8	32	33	58	48	11	37	13
	9	28	26	48	39	38	31	11
	11	15	49	42	56	16	34	5
	16	27	50	36	60	61	38	8
Total	202	329	473	569	562	479	351	132
Mean	16.83	27.42	39.42	47.42	46.83	39.92	29.25	11.00
Standard error	3.77	4.35	5.04	2.83	2.80	6.64	1.75	1.11

Table 29. Effect of buffer pH: Results of Duncan's Multiple Range Tests (cf. Figure 9 in the text)

	pH	Mean	Designation*
Exp. I	7.5	45.5	a
	7.0	44.3	a
	8.0	42.5	ab
	6.5	37.9	abc
	8.5	30.1	bcd
	6.0	27.8	cde
	5.5	22.8	de
	9.0	16.2	e
Exp. II	7.0	47.4	a
	7.5	46.8	a
	8.0	39.9	ab
	6.5	39.4	ab
	8.5	29.3	bc
	6.0	27.4	cd
	5.5	16.8	de
	9.0	11.0	e

* Treatment means not having a common letter in their designation are significantly different at the 5% level of significance.

Table 30. Lesion counts: Dilution curve for the purified preparation of the vetch isolate of clover yellow mosaic virus Exp. I (cf. Figure 10 in the text)

	CYMV concentration (µg/ml)			
	1	2	4	6
Lesion counts/ half leaf	8	41	73	69
	39	46	51	68
	22	52	46	91
	31	31	53	85
	21	26	65	97
	18	32	66	92
	14	19	69	108
	10	15	54	103
	23	16	41	106
	19	47	36	67
	15	58	32	77
	12	18	58	87
	11	42	57	74
	24	30	71	70
	28	10	35	80
	37	23	33	95
Total	333	506	810	1369
Mean	20.75	31.63	52.50	85.56
Standard error	2.34	3.65	3.52	3.49

Table 31. Lesion counts: Dilution curve for the purified preparation of the vetch isolate of clover yellow mosaic virus Exp. II (cf. Figure 10 in the text)

	CYMV concentration (µg/ml)			
	1	2	4	6
Lesion counts/ half leaf	41	58	56	98
	36	51	61	101
	29	33	47	96
	51	31	93	113
	20	42	71	111
	18	45	90	158
	9	38	76	106
	4	29	65	105
	21	53	78	108
	23	25	72	102
	35	28	51	98
	38	32	62	92
	40	48	47	109
	7	40	81	152
	16	43	89	145
	13	47	85	149
Total	401	643	1124	1843
Mean	25.06	40.18	70.25	115.18
Standard error	3.48	2.46	3.99	5.55